ENUMERATION AND GENETIC ANALYSIS OF COLIFORM BACTERIA FROM RIVERINE SYSTEMS OF ROURKELA: AN INDEX OF WATER POLLUTION

Dissertation submitted in partial fulfillment of the requirements for the degree of

Master of Science in Life Science

By

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CERTIFICATE

This is to certify that the project report titled **"**Enumeration and Genetic analysis of Coliform Bacteria from riverine system of Rourkela: An index of Water Pollution" submitted by Ms Shalini Srivastava to the department of Life Sciences, National Institute of Technology, Rourkela in partial fulfillment of the requirements for the degree of Masters of Science in LIFE SCIENCES is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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DECLARATION OF CANDIDATE

The work embodied in this report is an original investigation carried out by me, on the topic entitled, “Enumeration and Genetic analysis of Coliform Bacteria from riverine system of Rourkela: An index of Water Pollution”, for partial fulfilment of degree in Master of Life Science, NIT Rourkela. To the best of my knowledge and belief, this work has not been submitted to any other University or Institution to confer any Degree or Diploma.

Date:

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SHALINI SRIVASTAVA

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III. ABSTRACT

The coliform group has been used extensively as an indicator of water quality and has historically leaded to the public health protection. The aim of this estimation is to examine methods currently in use or which can be proposed for the monitoring of Coliforms in drinking water. Purposely, the need for more rapid, sensitive and specific tests is required to know the bacterial population in domestic water. Most acceptable methods for coliform detection include the multiple-tube fermentation (MTF) technique and the membrane filter (MF) technique using different specific media and incubation conditions. These methods have some limitations, however, such as in the time of duration of incubation, antagonistic organism interference were observed. Nowadays, the simple and inexpensive membrane filter technique is the most widely used method for routine enumeration of Coliforms in domestic water water as wellas MF method is best suitable method to identify the coliform in domestic water area. The detection of Coliforms based on specific enzymatic activity have improved the sensitivity of these methods. The enzymes are beta-D galactosidase and beta-D glucuronidase are widely used for the detection and enumeration of total Coliforms and *Escherichia coli,* respectively. Many fluorogenic substrates exist for the specific detection of these enzymatic actions, and various profitable tests based on these substrates are available. Frequent comparisons have shown in these tests may be a suitable alternative to the conservative techniques. They are more expensive, and the incubation time, even though cheap, remains take too long time for same-day results. Detection of Coliforms by molecular methods is also proposed, as these methods allow for very specific and rapid detection without the need for a cultivation step. Molecular-based methods are evaluated here by the process by the polymerase chain reaction (PCR). In the immunological method, different antibodies against coliform bacteria have been formed, but the application of this technique often show low antibody specificity. PCR can be used to distinguish coliform bacteria by means of signal amplification. DNA sequence coding for the beta-galactosidase gene and the beta-D glucuronidase gene has been used to detect total Coliforms and *E. coli* respectively.

1. INTRODUCTION

Environmental pollution, a potential worldwide problem, has rendered waters along the coastline and recreational beaches unsatisfactory for public use. Population explosion and insufficient infrastructure to properly treat and dispose of the manure, lack of sanitary condition, poverty and over exploitation of natural water has resulted in the discharge of considerable quantities of untreated waste chuck into the natural waters. This organic pollution is especially severe in the coastal waters due to the large density of population in coastal areas (Scialabba, 1998). Direct release of domestic waste and leaching from poorly maintained the septic tanks and improper management of farm waste are implicit as the major sources of waterborne diseases (Huttly, 1990). Sewage effluent has a wide range of pathogenic microorganisms which may pose a health hazard to human population, when they are discharged into the boring waters (Borrego and Figueras, 1997). Coliform bacteria, as typified by *Escherichia coli* and fecal matter *streptococci (enterococci*), have for decades been used for indicator organisms. The indicator organism is a microorganism whose presence is confirmation that water has been polluted with the feces of humans or other warm blooded animals like human. The coliform bacteria's group, commonly used as an indicator, it is defined as the aerobic and facultative anaerobic, nonsporing, Gram negative rods that ferment lactose with gas production within 48 hours of incubation at 35°C. Coliforms are present in the intestinal tract, and are excreted more numbers in feces matter, averaging about 50 million coliforms per gram in faces. Pathogenic bacteria and viruses causing enteric diseases in humans obtain from fecal discharges of infected persons. Fecal coliform's levels in the mainstream of the river varied considerably by time of year, and generally showed a pattern of either being low to moderate and stable throughout the study area, declining from up to down in river, or increasing from upriver to downriver. One of the main character of the indicator organism (coliform bacteria) is that it must be present at a higher concentration than the pathogens. For this reason, methods that can discriminate the source of coliforms may have greater predictive and useful value, compared to developing multiple tests that must target specific pathogens. It would be useful to identify the source of fecal pollution through regular water analysis, so that probable remediation efforts can be more focused and efficient. Many attempts have been made to develop methods that differentiate sources of fecal contamination,

including the use of fecal *Streptococci.* Initially, the ratio of fecal coliform to fecal *streptococci* was used as an indicator of fecal source. Virulence factors of pathogenic bacteria (adhesins, toxins, invasins, protein secretion systems, iron uptake systems etc) may be encoded by particular regions of the prokaryotic genome termed Pathogenicity islands (PAI). Pathogenicity islands were first described in human pathogens of the species is *Escherichia coli*, but has recently been found in the genomes of a variety of pathogens in humans, animals, and plants. Pathogenicity islands comprise large genomic regions [10–200 kilobases (kb) in size] that are presents on the genomes of pathogenic strains but absent from the genomes of nonpathogenic members of the same or associated species. The finding that the GC content of pathogenicity islands often differs from that of the rest of the genome, and they present of direct repeat at their ends, the organization of pathogenicity islands with transfer RNA genes, the presence of integrase determinants and other mobility loci, and their genetic instability dispute for the generation of Pathogenicity islands by horizontal gene transfer, it is a process that is well known to contribute to microbial evolution. PAIs(Pathogenic Island) in pathogenic were first described in *E. coli*, it was soon discovered that pathogenic bacteria of species which was other than *E. coli*, both type of bacteria gram positive and gram negative, have in their genomes DNA segments which contribute to many of the below mentioned features of PAIs. Pathogenicity islands bring genes encoding one or more than one virulence factor. It was first described in human pathogens but it is also present in plant pathogens such as *Pseudomonas syringae* (O'Brien, 1998). One should keep in mind that in some pathogenic bacteria virulence factors contribute to pathogenic prospective, whereas in other, nonpathogenic bacteria the same factors may be important for survival and replication in demanding ecological niches, where they do not generate any pathogenic latent (like the iron uptake system in pathogenic *Yersinia* species and nonpathogenic *E. coli*) (Heesemann, 1999). In nonpathogenic hosts, these DNA segments may act as vigor islands or ecological islands rather than as PAIs. PAIs are present in the genomes of pathogenic organisms but absent from the genomes of nonpathogenic organisms. PAIs were first described as chromosomal DNA regions, but the increasing amount of sequence figures from extra chromosomal elements supports the view that PAIs may be also part of plasmids or bacteriophage genomes. PAIs absorb relatively great genomic regions. The more of PAIs cover DNA regions of 10–200 kb. Strains of a variety of species, though, may also carry insertions of small pieces of DNA which may indicate the virulence factors. These region of DNA have been

termed pathogenicity islets. PAIs are frequently flank by small directly repeated (DR) sequences. These sequences may be produce after integration of PAI-specific DNA regions into the host genome via recombination. PAIs are often linked with transfer RNA (t RNA) genes. Transfer RNA loci always act as integration sites for foreign DNA and the association of PAIs and tRNA loci may therefore reflect the generation of PAIs by horizontal gene transfer. PAIs often carry hidden or functional genes which encoding the mobility factors such as integrases, transposons, and insertion sequence (IS), elements or parts of related elements. PAIs often does not represent homogeneous pieces of DNA but rather are made up of mosaic-like structures which have been generated by a multistep process. PAIs often represent in unstable DNA regions. Deletions of PAIs may occur through the direct repeats (DRs) at their ends or using IS elements or other homologous sequences located on PAIs. Additionally, a few PAIs [e.g. the high PAI (HPI) of *Yersinia pseudotuberculosis* have the capacity to move from one tRNA site to another site (Kaper, 1999), and other PAIs may be mobilized and transmitted by bacteriophages (e.g. those of *Staphylococcus aureus* and *V. cholerae*) (Kaper, 1999 and Novick, 2001). Particular PAIs represents the integrated plasmids and conjugative transposons, or bacteriophages or part of such elements.

1.1. Occurrence of Pathogenicity Islands

PAIs take place in the genomes of various human, animal, and plant pathogens. Enterobacteriaceae (like *E. coli, S. flexneri, S. enterica*, and *Yersinia* spp.) cause either intestinal or extra intestinal infections through virulence factors encoded on PAIs. Virulence factors encoded on PAIs which is represent the complete range of bacterial virulence factors, from adhesion to toxins to host defense avoidance mechanisms.

1.1.1. Adhesions

PAIs located in the genomes of various species and pathogen types encode adhesions, which mediate the capacity of microbes to attach the specific receptor molecules of eukaryotes. Thus, P fimbriae, which represent important adherence factors of UPEC, are encoded by UPEC-specific PAIs (Goebel et al., 1999). These attachment factors have the capacity to bind to galactose 1-4–galactose-specific receptor molecules on uroepithelial cells. In addition of UPEC or meningitis causing *E. coli*, have the ability to produce *S fimbriae*, which is bind to Sialic acid-specific receptors on uroepithelial cells and on brain cells (Hacker et al., 1994). *S-fimbria*-specific genes

(sfa) are part of a PAI that also carry genes for the iron uptake system, which was initially found in *Salmonella* spp.

1.1.2. Secretion Systems

Five different mechanisms for extracellular secretion of proteins, known as types I, have been describe in gram-negative bacteria, such secretion mechanisms are necessary for the extracellular secretion of virulence factors to the surface of the host cell or direct translocation into the host cell. Extracellular secretion of protein via type II, IV, and V mechanisms requires the instantaneous general secretion pathways, whereas proteins were secreted via type I and III mechanisms do not require the instant system. Type III and type IV systems are the mechanisms that are most closely associated with PAIs, even though types I, II, and V can also be found on PAIs.

1.1.3. Type III Secretion Systems

Type III secretion systems have been found deceitful on PAIs in the *Salmonella*, *Yersinia*, *Shigella*, EPEC, and EHEC and also in plant pathogens like *P. syringae*, *Erwinia* spp., *Xanthomonas campestris*, and *Ralstonia solanacearum* (Hueck, 1998; Collmer, 2000). Type III systems are not found on nonpathogenic members of these species. For most of these PAIs, genes encoding the type III secretion system and genes encoding the proteins secreted by the type III system include the great majority of the genes enclosed on the PAI. Although the sequence and host cell activities of the secreted proteins be different according to the individual pathogen, the majority of the less 20 proteins that comprise the type III secretion apparatus be highly conserved among the different pathogens. Thus, PAIs of several mammalian and plant pathogens encode type III secretion systems that are vital for virulence.

1.1.4. Type IV Secretion Systems

Like type III systems, type IV secretion systems have also been directly associated with PAIs. Type IV systems have been present which is important for full virulence of large number of pathogens, like *H. pylori*. *H. pylori* has the type IV system encoded on the Cag PAI has recently been shown to mediate the translocation of the Cag A protein to host cells. Cag A plays an important role in the initiation of host cellular growth changes induced by *H. pylori*. In *B. pertussis*, the type IV secretion system is necessary for secretion of pertussis toxin in to mammalian cells. The type IV secretion system, first observed in the plants pathogen where in it mediates the transfer of DNA into the plant cell. Type IV system in this species is prearranged on

the 200-kb Ti plasmid, which is essential for crown gall tumor genesis of higher plants (Winans et al., 1999). Ingredient of the Ti plasmid called T-DNA; it is transferred into the plant cells, where it is integrated into the host genome.

1.1.5. Type V Systems

Type V secretions systems (T5SS) was also referred to as auto transporters. After proteolytic cleavage of the signal sequence, the carrier domains of pro protein oligomers form a barrel structure in the outer membrane and the fare domain of the proprotein passes through the pore formed by the barrel. Finally, proteolytic cleavage allows the release of the commuter domain into the extracellular space. There are different commuter domains secreted by T5SS, e.g., the immunoglobulin G (IGg) proteases and also the VacA toxin. Examples of T5SS encoded by PAI which are LPA and the "*Esp C"* PAI of the pathogenic *E. coli*.

1.2. Protein secretion systems encoded by PAI

Secretion of proteins is a general essential for pathogenic and nonpathogenic bacteria. It Secreted proteins which are compulsory for the assemblage of the cell envelope, metabolism, and defense system, and the interaction with the host cells during pathogenesis. In gram-positive bacteria, surface protein and extracellular protein are secreted by the extensive secretion pathway. In difference, the presence of an outer membrane in gram-negative bacteria leads to the evolution of a remarkable variety of structurally and functionally diverse secretion systems.

1.2.1. Structure of PAI

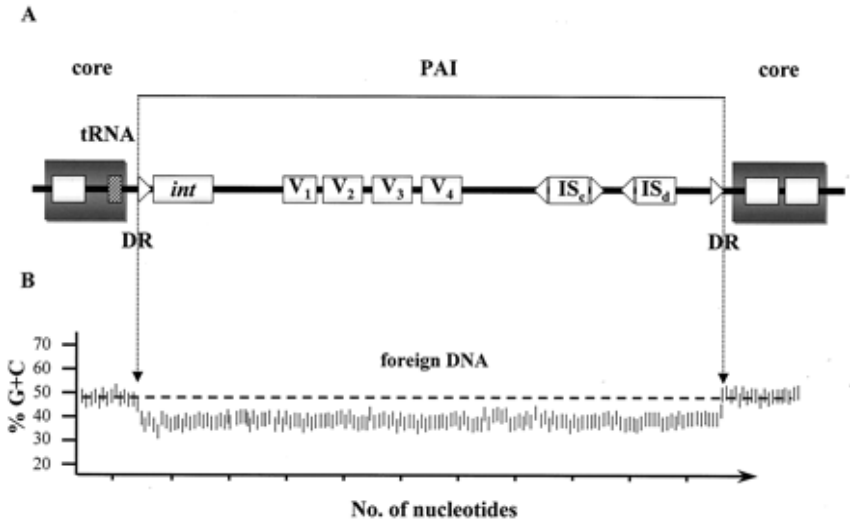


Fig.1.1. Structure of PAI

PAI are mostly inserted in the spine genome of the host strain (dark grey bars) in specific sites that are frequently tRNA or tRNA-like genes. Mobility gene, such as integrases (*int*), it is often located at the beginning of the island, it's close to the tRNA locus or the respective attachment site.

1.2.2. Main Features of PAI

(i) PAI carries one or more virulence genes; genomic elements by means of self similar to PAI but lacking virulence genes are referred to as genomic or metabolic islands.

(ii) PAI is present in the genomes of a pathogenic bacterium but absent from the genomes of a nonpathogenic agent of the same species or a closely related species.

(iii) PAI occupies moderately large genomic regions. The majority of PAI have the range of 10 to 200 kb.

(iv) PAI often differs from the middle genome in their base composition and also shows a different codon custom. The base composition is expressed as percentage of guanine and cytosine (GC) bases, and the average GC content of bacterial DNA can range from 25 to 75%. Nearly all pathogenic bacterial species have GC contents between 40 and 60%.

(v) PAI is often located adjacent to tRNA genes. This inspection gave rise to the hypothesis that tRNA genes provide as attach points for insertion of foreign DNA that has been acquired by horizontal gene transfer. The frequent insertion on tRNA loci may be explained by the observation that genes encoding tRNAs are highly preserved between various bacterial species.

(vi) PAI is frequently attached with mobile genetic elements. It is often flank by direct repeats (DR). DR is defined as DNA sequences of 16 to 20 base pair (up to 130bp) with a perfect or almost perfect sequence repetition. PAI often carry hidden or even functional mobility genes such as integrases or transposases. Integrases, which may have been obtained from lysogenic bacteriophages, arbitrate the combination of the phage genome into the bacterial genome, as well as the elimination of genome needed to enter a lytic cycle. These mobile genetic elements can change their location inside the chromosome, but transposons also jump from a chromosomal location into a plasmid and vice versa. Insertion sequence (IS) elements are commonly observed in PAI. Insertion of IS elements can effect in the inactivation of genes, but the grouping of two or more IS elements can also result in the mobilization of larger portions of DNA. PAI can also represent conjugative transposons, integrated plasmid, bacteriophages and parts of these elements.

(viii) PAI often represents mosaic-like structures rather than homogeneous segments of horizontally acquired DNA. Several PAI symbolize an insertion of a single genetic element. Others show an extra complex structure, while elements of different origin are present. PAI, which are the best, understood genomic islands recognized to date, carry clusters of virulence genes whose products give to the pathogenicity of the bacteria. In of *E. coli*, such islands have allowed the bacteria to adjust to specific environment and to cause disease. The division of fitness islands into the different subtypes is based not only on their genetic composition but also on their effects in a specific ecological niche and inside a particular organism.

Coliform bacteria have large grouping of a variety of species of bacteria. Some members of this group of microbes are originating in natural environmental soils, surface water and plants. Other types of coliform bacteria survive in the intestines of humans and other warm-blooded animals and are characteristically near in the discharged fecal material from the host organism. In this group of bacteria that is of impact during the analysis of water because, it is used as indicator of bacterial pollution in domestic water. Food or water sample in which this group of bacteria is present has potentially come in contact with animal manure, domestic sewage, contaminated soil, plant and animal material. External the host, bacteria expire rapidly, normally within 30 days. So, if coliform bacteria are seen in a well in excess of a time consuming period of time, it may be observed that new bacteria are entering the well or aquifer (natural underground water supply). It follows that such a water supply which has pathogenic bacteria and viruses, which cause the many infectious disease, grave human illnesses like (typhoid fever, hepatitis, dysentery) etc. The presence of coliform bacteria shows the possibility, but not a confidence, that disease organisms may also be near in the water. When absent, there is a very low probability of disease from the water. The ability of the total coliform test to dependably predict the bacterial safety of water relative to the hundreds of possible diseases that might be current is critical since it impossible to check separately for every disease organism frankly on a monthly or quarterly basis. Recently however, public health experts have known that certain protozoa, which cause disease, such as giardia and cryptosporidium, can be present in surface water still when the total coliform test shows absence. While an important exception, the total coliform test remains the normal for determining the bacterial quality of drinking water.

1.3. Evolution and transfer of PAI

The virulence factor are present in very similar forms in different bacteria, it is explain by gene transfer horizontal. Different scenarios can be considered to explain the transport between bacterial strains and species.

1.3.1. Natural Transformation

Certain bacteria are capable of natural transformation. For the certain growth phase period, transport systems are expressed that allow the uptake of environmental free DNA. Even though the majority of this foreign DNA will be degraded, some fragments that port "useful" genes are integrated into the genome of the beneficiary and maintained. It appears possible that this method allows uptake of DNA from faintly related species that will be maintained as the selective strain selects for the newly acquired features.

1.3.2. PAI and Plasmids

Similar clusters of virulence genes are present in PAI and on virulence plasmids, representing that episomal and chromosomal locations are possible for the identical gene cluster. It was observed that definite clusters of virulence genes are present in PAI of some pathogens but also on virulence plasmids in other bacteria. The T3SS requisite for offensive of epithelial cells by *Shigella* spp. it is encoded by the mxi and spa genes located on a virulence plasmid, and a related gene cluster that is necessary for the invasiveness of *Salmonella enterica* is located in SPI-1 in a location of chromosom. Conjugation may allow the transfer of plasmids between bacteria. These plasmids then replicate an separately in the bacterial chromosomes, but under definite conditions plasmids can also integrate into the chromosome of organisms. On the other hand, the formation of episomal elements has been reported in certain PAI of the *Staphylococcus aureus*. Thus, the plasmids could be another means of transfer of PAI between bacteria.

1.3.3. Transduction

Bacteriophages have been isolated from practically all bacterial species; even obligate intracellular pathogens like as *Chlamydia* spp, which contain specific phages. Bacteriophages having ability for transfer of bacterial virulence genes in to their genomes. Sometimes transfer of virulence genes by phages produce bacteria to colonize or establish in new habitats, such as new host organisms or exact anatomic sites. This extension also allows a more capable spread of the bacteriophages. Therefore, the transfer of bacterial virulence genes as passengers in the viral genome can also be evolutionary earnings for the bacteriophage. PAI does not occur only in

human pathogens; they also found in animal and plant pathogen. Examples are *Pseudomonas syringae* and *Xanthomonas campestris* and islands in animal; *salmonellae* and *staphylococci.* They are spread all through the bacterial globe, and horizontal transfer may be facilitated by plasmids and phages or by bacteria, which are vigorous for the uptake of free DNA by natural transformation.

E. coli mainly effect on infected the mucosa of the stomach, an appendage that has long been considered an environment too antagonistic for bacterial colonization. Infections with *H. pylori* are general and are often acquire in early days, and sensitive infection can produce to chronic colonization of the gastric mucosa (Wolff et al., 1998). This migration usually leads to chronic gastritis, and succeeding forms of disease are dependent on host as well as on bacterial factor However, patients with low or high invention of gastric acid can grow gastric ulcer or duodenal ulcer, respectively. There is also a strong association between infection by *H. pylori* and development of mucosa-associated lymphoid tissues lymphoma and gastric cancer, resulting in the categorization of *E. coli* as a carcinogen. *E. coli* organisms are curved, rod in shaped bacteria with a group of polar flagella and are covered by a membrane sheath. Motility is a very important virulence factor and enables the bacteria to break through the mucin layer of the gastric epithelium (Josenhans et al., 2002). This bacterium also produces urease. This enzyme catalyzes the formation of CO_2 and ammonia that can counterbalance the acidic pH in the locality of the bacteria. Development of *E. coli* requires a micro aerophilic atmosphere and complex media. Clinically isolation of *E. Coli* have been classified into type I and type II strains, which are linked with different clinical outcomes ranging from gastric ulcer to asymptomatic migration. There are also various forms of intermediary virulence. Type I strains carry genes programming both, the cytotoxins *CagA* and *VacA*, while type II strains contain VacA genes only (Covacc et al., 1995). VacA is a secreted toxins that induces extensive vacuolation in epithelial cells, cell deaths, and obliteration of epithelial integrity.

The attachment of type I strains to gastric epithelial cells generate the synthesis and secretion of several chemokineses, and the secretion of interleukin-8 (IL-8) is frequently assayed in replica systems. It has been observed that the infection of epithelial cells by *E. coli* leads to dramatic rearrangements of the host cell actin cytoskeleton and the formation of pedestals (Tomb et al., 1997) that are indicative of EPEC induced pedestals, as well as to alter in the gross morphology of host cells (humming bird phenotype). These phenotypes are associated with alteration in the

signal transduction pathways of the host cell and the presence of a tyrosine-phosphorylated protein (Falkow et al., 1999). Detailed analysis of the *cagA* loci in type I and type II strains indicated that the final group showed deletions of a large chromosomal section. This locus had the typical characteristics of a PAI and was termed the cag PAI (Censini et al., 1998) characterized this locus and showed that the *cag* PAI had a size of 37 to 40 kb, flank by direct repeats of 31 bp (Fig. 1.2).

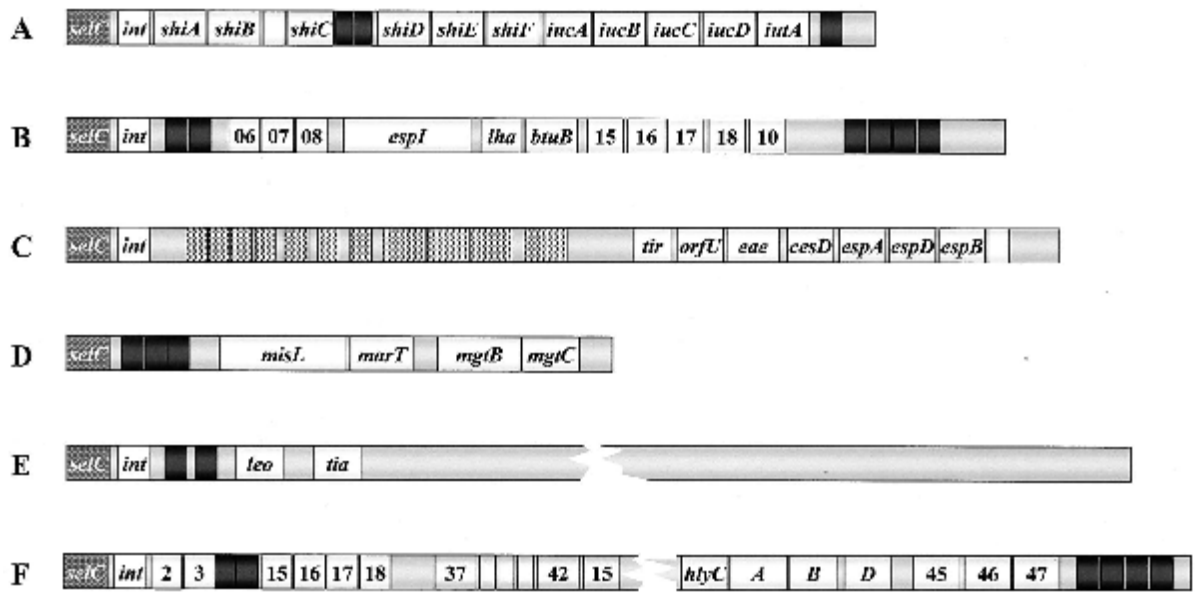


Fig.1.2. Comparison of various PAI integrated at the *selC* locus.

The locus has a GC content of 35%, in distinction to the 39% observed for the center genome. A gene for a tRNA has not been recognized at the dot of integration, but the *glr* gene (glutamate racemase) was disturbing by insertion of the PAI. There are no genes linked with DNA mobility within the cag PAI of type I strains. Nevertheless, the presence of an IS *605* element within the *cag* PAI of strains with transitional virulence phenotype was observed. In strains of transitional virulence, various forms of deletions with the cag PAI were detect, and in positive strains the locus was separated into two portions, referred to as cagI and cagII (Covacci, 1996 and Berg, 1998). These observations support a correlation between the presence and integrity of the cag PAI and the brutality of disease. Studies with a mouse model have shown that an association between cag PAI-negative *H. pylori* strains and cag PAI-positive strains that are mouse adapted

and have modulate their ability to activate a inflammatory response can better colonize than the parental strain , indicating that the cag PAI of type I strains may turn into lost during migration of infected animals. In addition to large deletions and chromosomal rearrangement of the cag PAI, there are indication that point mutations in the PAI genes result in alteration of CagA translocation and IL-8 induction. This effect can be explained by loss of function of the T_4SS. After translocation, CagA is tyrosine phosphorylated and induces growth factor like phenotypes charecter in the host cell. SHP-2 (SRC homology 2 domain [SH2]-containing tyrosine phosphatease) was recognized as a cellular target of CagA. Activation of SHP-2 by CagA might give to the irregular proliferation and movement of gastric epithelial cells, thus causative to the pathogenesis of *H. pylori* in gastric infections. It has also been projected that phosphorylated CagA may generate the transcription of nuclear genes.

The topology of PAI of various pathogens is depicted to express different properties of PAI (Fig. 1.3). The functional classes of the genes are indicated in the figure: (A) the cag island of *E.coli* genes used for a type IV secretion system (T_4SS) (grey symbols) that can arbitrate the translocation of the effectors protein CagA (dark grey) into eukaryotic cells. (B) *Salmonella* SPI-2 has an assortment structure. It has been defined as a genetic element of about 40 kb that is absent in nearly related species of *E. coli.* Only a 25 kb portion is required for systemic infection and encodes a T_3SS system (grey), secreted proteins (dark grey), and regulatory proteins (white). Another portion (15 kb) is not essential for virulence and port genes for metabolic or indefinite functions (light grey symbols), such as an enzyme system for deputy electron acceptors during anaerobic growth. Genes associated with mobility are indicated as dark dotted symbols. (C) The HPI of *Y. enterocolitica* is an example of an uneven PAI. Numerous is elements are present within this PAI (dotted arrows). Genes in HPI encodes a high affinity iron uptake systems (dark grey) that is most important for the extracellular proliferation of the pathogen during colonization of the host. (D) The Sal PAI of MRSA is shown. A notable feature of PAI in *S. aureus* is the presence of a huge number of genes with connected functions, such as genes for enterotoxin (dark grey) or lipoproteins (grey).

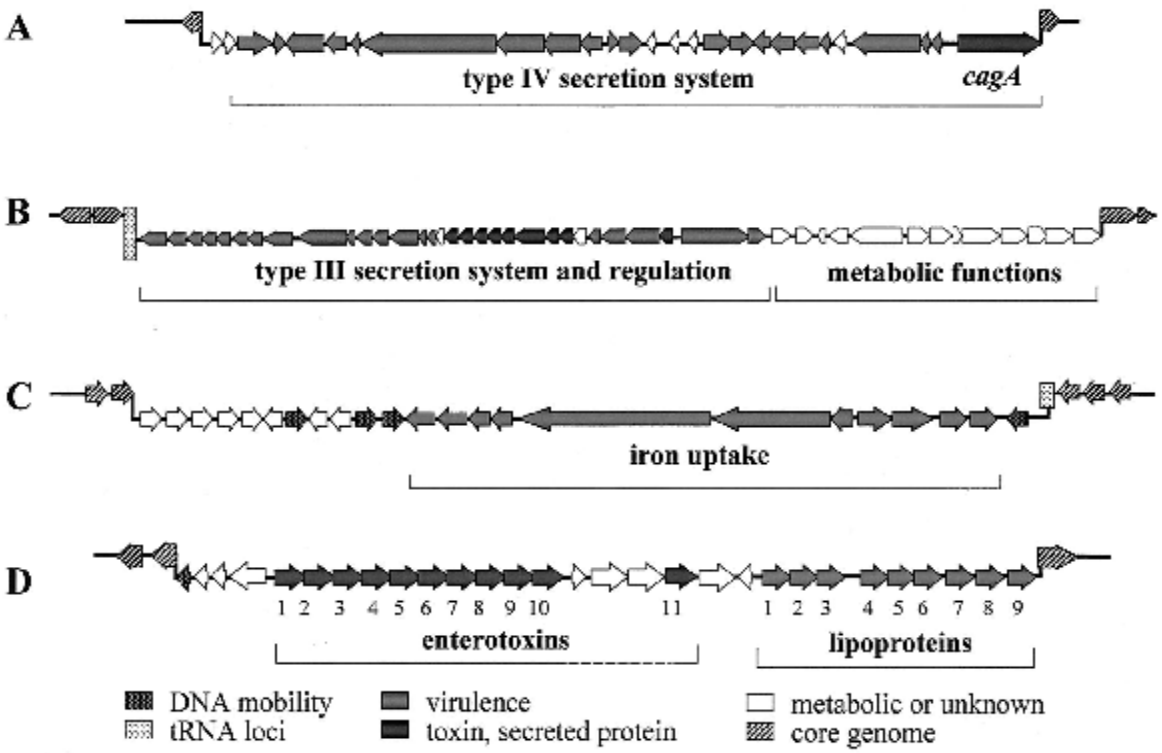


Fig.1.3. Examples of PAI of various pathogens

1.4. Bacterial Pathogens without presence of PAI

In the direction of our current information, PAI are absent in *Mycobacterium* spp., *Chlamydia* spp., the *spirochetes*, most *streptococcal* species, and quite a few other pathogens. The reasons for the absence of PAI in certain species have not been well understood, but comparison of the life-style of pathogens with and without PAI might give some hints about the scheme of primary principles. Some groups of pathogens lacking PAI show an acute adaptation to a specific host environment that is also convoy by reduction of the genome size and loss of the ability to replicate outside a host. In feature, most pathogens harbor PAI shows a high degree of flexibility in the consumption of different host or body sites of a host for their proliferation. In addition, pathogens containing PAI are often able to exist in natural environments. These observations suggest that PAI expand the range of habitats that can be colonized by a bacterial species. The observation that metabolic function can also be found in genomic islands supports the idea that acquirement and maintenance of these elements allows the entrance to new habitats. Therefore, highly adapted and particular pathogens develop in the overturn direction, *i.e.*, toward loss of flexibility. The specialization is given by genome reductions, and it is possible that such a

reduction lead to the removal of major portions of horizontally acquired DNA elements. Extreme regulation of a parasitic lifestyle might also result in reduced contact to the microbial gene pool. For example, obligate intracellular pathogens have misplaced mechanisms that allow genetic exchange by conjugation or natural capability for the transformation process. However, even highly modified pathogens such as *Chlamydia* spp. have specific bacteriophages (Bavoil, 2000) that strength gives to a certain extent of horizontal gene transfer. Absence of PAI level shows a high degree of flexibility in a host assortment and in a free-living life-style, such as most streptococci. A reason of this observation might occupy the high velocity of recombenent in this group of bacteria. One can consider that horizontal gene transfer also plays vital role in these pathogens but that the express recombination may make the identification of horizontally acquired DNA complex. The absence of PAI in most streptococcal species could be explained by the high rate of genome reorganization in these bacteria, resultant in mosaic chromosomes and mosaic gene structures (Saizieu, 2001). The existence of mobile genetic elements in ***streptococci*** indicates that the insertion of large blocks of virulence genes may also get position. It has been estimated that 10% of the total genome of *S. pyogenes* consists of bacteriophages and transposons (Saizieu, 2001). The rapid genomic rearrangements in ***streptococci*** may result in separation of horizontally acquired gene blocks neutralize the appearance of typical PAI. In conclusion, uptake of large fragments of DNA containing virulence genes is likely to take place in the mass of bacterial pathogens. The mode of recombination of foreign DNA into the host chromosome may decide whether or not a visible PAI is formed.

2. REVIEW OF LITERATURE

This study was mainly based on isolation of coliform bacteria in ground water and river since bacterial contamination cannot be detected by flavor, aroma, or view. All drinking water wells should be tested at least once a year for coliform bacteria. Michigan's well construction code require all new, repaired, or reconditions wells to be sanitary with chlorine to kill bacteria that may have been introduced during construction. Testing was required initially to cover that the water is free of coliform bacteria before the well is put into examine. A coliform bacteria test is also suggested immediately if:

- A sudden changes occur in water's taste, sight, or odor.
- The water turns gloomy after rainfall or the top of the well was flooded.
- Suspect a contamination source (e.g., septic system or barnyard) is within 50 feet of well.

Water sampling containers can be obtained from Washtenaw Country Environmental Health. Be sure to use proper sampling techniques while taking the sample, as human blunder in sampling can cause false positive sample results. Refer to the directions that fleet the water sample pot. A clean, well maintain and frequently used valve should be used to collect the samples. Coliform bacteria do not always seen in every sample. They can be show sporadic, patchy and sometimes seasonal when they occur in a water supply. May be get disturbed but do not panic if coliform bacteria are detected. Before treat, repair, or replacing in well, it is clever to resample at once if a positive sample is collected. If you receive a second positive sample for total coliform, or if the initial sample is positive for fecal Coliforms, does not consume the water. Take the water to a continuing boil for three minutes to kill the bacteria. Coliform bacteria are commonly found in top soil, on foliage, and in plane water. They also live in the intestines of warm blooded animal and human. Some coliform bacteria strains are able to survive in soil and water for long periods of time. Coliform bacteria will not likely cause sickness. Though, coliform bacteria are most commonly associated with manure or plane waters, the presence of coliform bacteria in drinking water indicates that disease causing organisms (pathogens) may be found in the water system. There are diverse groups of coliform bacteria, each have shows a different stage of risk. If Coliform bacteria are present, the source of the problems must be identified. Routine test from several locations within the water systems are helpful to identify. The complete water systems

might be methodically flushed and disinfected before negative bacteria samples can be obtained. Sometimes it is necessary to depart over the disinfection process.

A well drilling contractor or local health department sanitarian can help to identify the structural defects in the water system. These includes: openings at the top of the well, old, rusty, or damaged well casing, unprotected suction line, buried wellhead, and close proximity of a well to septic tanks, drain fields, sewer, kitchen sinks, drain, privies, barnyards', animal feeds, dumped wells, and surface water area. Cross connections with waste water plumb can also be introduce coliform bacteria into the water supply system. If any of the above conditions were found, then proper changes or repairs should be arranged. After the defects were corrected to the entire water system should be disinfected and the water tested before drinking. Population sudden increase and inadequate infrastructure to properly treat and dispose of the sewage, lack of sanitary surroundings, poverty and over exploitation of natural water has resulted in the discharge of substantial quantities of untreated waste into the natural water. These organic pollutants are especially cruel in the coastal water due to the large density of residents in coastal areas (Scialabba, 1998). There are the following conditions: direct discharge of domestic wastes, leaching from badly maintained septic tanks, and offensive management of farm waste were suspected as the major sources of waterborne diseases (Huttly, 1990). Sewage waste matter contains a wide range of pathogenic microorganisms which may cause a health hazard to human population when they are discharged into the recreational water (Borrego and Figueras, 1997) and the health hazard might be strict in a heavily colonized country such as India. In India, almost three quarters of a billion citizens live in rural areas without admittance to safe drinking water and water borne infections were a major cause of motality (Patil et al., 2002). Disease such as enteric fever and diarrheal diseases are highly endemic toward India and are major public health problems among the children under the age of five years observed. Faecal coliform, *Escherichia coli* (the predominant member of faecal coliform group) an operationally defined grouping of enteric bacteria whose occurrence in natural waters is used as an pointer of recent fecal contamination and therefore, the probable presence for pathogenic microbes (Rhodes and Kator, 1998) other than their absence does not necessarily assurance quality of water (Dutka, 1973). *E. coli* include numerous pathogenic serotypes such as enterotoxigenic *E. Coli* (ETEC), enteroinvasive *E. Coli*(EIEC), enterohemorrhagic *E. Coli*(EHEC), enteropathogenic *E. Coli* (EPEC) enteroaggregative *E. coli* (EAgEC) and enteroadhesive *E. coli* (DAEC), which are of

public health inference worldwide and are a chief cause of sensitive diarrhea in children in increasing countries (Nataro & Kaper 1998; Rodrigues et al., 2002). The existence of human pathogenic bacteria such as *Salmonella, V. cholerae, V. parahaemolyticus*, and pathogenic serotypes of *E. coli* has been reported from coastal area (Venkateswaran et al., 1989; Daniels et al., 2000; Hatha et al., 2004). The monsoon is a very important in once a year climatic feature of Kerala and hence the study period has been divided into three distinct seasons, such as pre-monsoon (February to May), present monsoon (June to September) and post monsoon (October to January). Seasonal difference of marker bacteria at every position during different seasons is required to estimate test. The results indicate that considerably higher levels of indicator bacteria in the southern part of the lake (enclosed) during the monsoon than the northern region were found higher bacterial population during these months may be due to the increased province run off during the monsoon period resulting in a higher fecal input into the lake from a mixture of sources. In these (Abhirosh and Hatha, 2005) earlier studies on the inactivation kinetics of indicator bacteria in Cochin estuary we could found that sunlight was a chief factor disturbing the self purifying capacity of the natural water. Reduced strength of sunlight due to cloudy conditions during the monsoon as well as increased turbidity from land run inedible resulting in reduced penetration of sunlight might expand the survival of faecal indicator bacteria. An amplify in the faecal coliform level after rainfall get sometime polluted the perticular region (Shehane et al., 2005). Conversely in the northern region (open) more active environmental conditions overcome (especially salinity) and the monsoon rains may dilute the bacterial load which enters into the lake. During December to March the system is clogged and natural flow is prevented which results in the gathering of organic load in the southern part of the lake, give proper environmental circumstances for the growth of bacteria. The use of this water for harvesting shellfish and enjoyment during this period may pose severe health risks to the citizens. After opening the salt water monitor in March, sudden increases in the MPN index of FC were observed at the time on the northern part of the salt water controller. During the summer months of March through May, tidal incursions at the northern part of the controller, however, resulted in a decrease in FC values perhaps due to the high salinity. The seasonal salinity on the northern part of the controller varied between 0–18 ppt and it have been report that the increasing in salinity causes sub toxic stress in *E. coli* and affects the continued existence of the bacteria (Anderson et al., 1979). The technique of enumerating Coliforms bacteria by resources of

multiple-tube fermentation (MTF) has been use for more than 80 years as a water quality monitoring technique. This method consists of inoculating a sequence of tubes with suitable decimal dilutions of the water samples. Production of gas, acid formation or abundant growth in the test tubes observed after 48 h of incubation at 37 °C compose a positive presumptive effect. Both lactose and lauryl tryptose broth can be used as a presumptive media, but (Seidler et al., 1981; Evans et al., 1981) contain obstruction, with a high figures of non coliform bacteria, use lactose broth. All tubes with a positive presumptive result are consequently subjected to a confirmation test process. The formation of gas in a brilliant green lactose bile broth (BGLB) ferment tube at any time within 48 hrs at 35°C shows a positive confirmation test. The fecal coliform test (use an EC medium) can be useful to find out total coliform population that is FC (APHA, 1998): the production of gas after 24 hrs of incubation at 44.5°C in an EC broth medium(determining coliform bacteria) is considered as a positive product. The outcomes of the MTF (Multiple Tube Filter) technique are articulated in term as most probable number (MPN) of microorganisms is presents. This number is a statistical estimate of the indicate number of Coliforms in the sample. As significance, this technique offers a semi-quantitative enumeration of Coliforms. However, the accuracy of the estimation is small and depends on the quantity of tubes used for the study: for example, if only one ml is examined in a sample containing one coliform per ml, about 37% of 1-ml tubes may be expected to acquiesce negative results because of the casual allocation of the bacteria in the sample. But, if in five tubes, each with 1 ml sample is used, a negative result may be expected less than 1% at a time (APHA et al., 1998). A group of factors may considerably affect a Coliform bacteria discovery by MTF, particularly through the presumptive period. Obstruction by high statistics of non coliform bacteria (Seidler et al., 1981; Evans et al., 1981; Means and Olson, 1981), as well as the inhibitory environment of the medium (McFeters et al., 1982), has been recognized as factors causative to underestimate of coliform profusion. The MTF technique lacks accuracy in qualitative and quantitative conditions. The time necessary to obtain outcome is higher than with the membrane filter technique that has replaced the MTF technique in numerous instances for the organized examination of drinking water. However, this technique residue useful, especially when the situation does not allow the use of the membrane filters technique, such as muddy or colored waters. MTF is easy to apply and it can be performed by a technician with basic microbiological instruction, but the technique can become very deadly and work hard intensive since countless dilutions comprise to be

processed for each water sample. Nevertheless, it is also comparatively inexpensive, as it requires primitive laboratory tools. However, this technique is particularly time consuming, requiring 48 hrs for presumptive outcome, and necessitates a subculture stage for confirmation which might obtain up and about to a further 48 h.

Vembanadu Lake indicates the regular discharge of sewage containing pathogenic microorganisms into the estuary and also the complete survival of these organisms are easily detectable at higher concentrations. The survival and determination of these bacteria in natural environments is of picky importance to public health as the population in this region is using this water body for numerous domestic purposes. This water body supports a major fish and shellfish assets and the presence of these pathogenic bacteria is still showing off a public health concern through related food borne outbreak. The increased occurrence of indicator microorganisms in the southern part of the lake may be due to the changed flood patterns due to the salt water valve. Conversely, the salt water regulator does not have any major impact on the occurrence of precise pathogens. The results of the present study concluded that the disinfected quality of this water is deprived and it may cause strict health risks to the population using the water for unusual purposes. The sterile quality of water is of the most significance to society, and enforcement of lawful events to organize entry of banned point sources effluents into this water body are necessary for good managing of this vital normal resource.

A frequent cross-sectional study were conducted to find out the patterns of antimicrobial conflict in *Escherichia coli* strain isolated from human leakage, natural fauna, domestic nature, farm environment, and surface water area. Isolation and identification of *E. coli* were ended by using enrichment media, selective media, and biochemical tests, antimicrobial susceptibility testing by the disk diffusion method was conducted for neomycin, streptomycin, chloramphenicol, trimethoprim, sulfamethoxazole, tetracycline, ampicillin, nalidixic acid, nitrofurantoin, cephalothin, and sulfisoxazole. Confrontation to at least one antimicrobial agent was demonstrated in isolates from domestic animals, buddy animals, human leaching, flora and fauna, and surface water. In general, *E. coli* isolates from domestic species show resistance to the largest figure of antimicrobial agents compared to isolates from human seepage, wildlife, and grond water. The agent to which resistance was confirmed nearly all habitually was tetracycline, cephalothin, sulfisoxazole, and streptomycin. There were similarity in the pattern of resistance in fecal samples and farm environment sample by animals, and the levels of Cephalothin resistant

isolates be higher in farm environment samples than in fecal samples. Multidrug resistance was visualizing in a multiplicity of resources, and the uppermost levels of multidrug resistant *E. coli* were observed for beast fecal samples. The information of water sample isolates were resistance only to cephalothin may suggest that the resistance patterns for farm environment samples may be more representative of the threat of contamination of surface water area with antimicrobial agent resistant bacteria. It has been recognized as an increasing worldwide problem in both human and veterinary medicine, and antimicrobial agent use as measured the most important factor for the appearance, selection, and distribution of antimicrobial agent resistant bacteria (Neu, 1992; Witte, 1998). The principle behind the progress of resistance is that bacteria in the guts of humans and animals are subjected to various types, concentration and frequencies of antimicrobial agents. Selective strain selects resistant bacteria that have precise for resistance to the antimicrobial agents that have been used (Prescott et al., 2000; Troy, 2002). There are four universal mechanisms of resistance, all of which are monitor by the action of specific genes, enzymatic inactivation or modification of antimicrobial agents, impermeability of the bacterial cell wall or membrane, active removal of the drug by the cell efflux pump, and alteration in target receptors (Prescott et al., 2000). Bacteria increase antimicrobial agent resistance genes during mobile elements are plasmids, transposons and integrons (Rubens, 1979; Prescott et al., 2000), which outcome in mutations in genes responsible for antimicrobial agent up take or binding sites (Spratt, 1994) or creation of portions of bacterial chromosomes (Hachler, 1991; Alekshun et al., 1999).Previously acquire, resistance genes can be transferred between bacteria and the ability of *Escherichia coli* to transfer antimicrobial drug resistance be well known (Richmond, 1972).

The term coliform bacteria cover a broad range of bacteria families and species, which belong to the family of enterobacteriaceae. These species and families differ significantly in terms of their pathogenic properties and virulence. The coliform group of bacteria is frequently used as an indicator; it is defined as aerobic and facultative anaerobic, nonspore forming bacteria, Gram-stain negative rods that ferment lactose with gas production within 48 hrs of incubation at 35^0C. Coliform exist in in the intestinal tract, and are excreted in huge numbers in feces, average about 50 million coliform per gram detected. Most coliform bacteria do not cause any particular disease, however some rare strains of *E. coli*, mainly the strain 0157:H7 can cause severe illness. Modern outbreaks of disease caused by *E. coli* 0157:H7 have generated a large amount of public

pain about this organism. *E. coli* 0157:H7 mainly found in cattle, chickens, pigs, and sheep. Most of the reports in human cases have been found due to eating under cooked hamburger. In case of *E. coli* 0157:H7 cause by contaminated drinking water supplies is ucommon. In human body there are various types of bacteria existing in many different parts of the body, and it usually do not causes any diseases in the host body. Similarly in the large intestine, or colon, is the major site for bacteria to present in a body. Approximately twenty percent of the faeces of an ordinary person consist of bacteria, most of which have approach from the colon. The main bacteria which are present in the colon are *Bacteroides*, *Bifidobacterium*, *Eubacterium*, *Coliforms*, *Streptococcus*, and *Lactobacillus*. They will not harm into the body it shows the positive communication in human body. The relationships between the human host and most normal flora usually fall under the group of Mutualism relationship. The advantage to the bacteria is that they get a place to survive and multiply it. The profit to the human host is as the host ability to support itself is to amplified. The bacteria may generate vitamins (such as B and K), and may split down food stuffs that are normally hard to digest by the host into mechanism that can be digested. Large quantity of fecal coliform bacteria in water is not harmful according to some establishment, but may indicate a higher threat of pathogens being present in the water systems (Fresno et al., 2009). Some waterborne pathogenic diseases that may overlap with fecal coliform contamination include: ear infection, dysentery, typhoid, viral and bacterial gastroenteritis and hepatitis A. The presence of fecal coliform tend to affect humans more than it does aquatic creature, while not absolutely .Most coliform bacteria do not cause any diseases, however, some rare strains of *E. coli,* mainly the strain 0157:H7, can cause serious illness. Pathogenicity islands are special class of genomic islands acquired by microorganisms through horizontal gene transfer mechanism. Virulence factors of pathogenic bacteria (adhesins, toxins, invasins, protein emission systems, iron uptake systems, and others types) may be determined by particular regions of the prokaryotic genome termed as Pathogenicity islands. Pathogenicity islands cover large genomic regions [10–200 kilobases (kb) in size] that are presents on the genomes of a pathogenic strains but absent from the genomes of nonpathogenic strains of the same or related species. PAIs take place in the genomes of various forms present in human, animal, and plant pathogens. A list of PAIs describe that several members of the Enterobacteriaceae family (e.g. *E. coli*, *S. flexneri, S. enterica*, and *Yersinia* spp.) And cause either intestinal or extraintestinal infections via virulence factors encoded on PAIs. Enterobacteria family as well as *V. cholerae, P. syringae,* and others,

show common gene transfer via plasmids and bacteriophages. Such extra chromosomal elements certainly give the impression to represent one source of PAIs (Hacker and Kaper, 1999). Classic examples are adherence factor, toxins, iron uptake systems, invasion factors and secretion systems. Pathogenicity islands are isolated genetic units flanked by direct repeats, insertion sequences or tRNA genes, which perform as sites for recombination into the DNA. Cryptic mobility genes can also be present, representative the origin as transduction. Pathogenicity islands (or PAIs) carry genes encoding one or more virulent factors including but not limited to adhesins, toxins, or invasins. They may be situated on a bacterial chromosome or may be transferred within a plasmid. The GC-content of pathogenicity islands repeatedly differs from that of the rest of the genome (Hacker and Kaper, 2000).

2.1. Regulation of PAI-encoded virulence functions

Like other virulence genes, PAI genes are usually not constitutively articulated but it respond to environmental signals. PAI are commonly part of complex rigid networks that include regulator encoded by the PAI itself, regulator encoded by other PAI, and global regulator encoded somewhere else in the chromosome or by plasmids. PAI regulators, in turn, can also be involved in the parameter of genes that are located exterior the PAI. Most commonly, regulators belong to the AraC/XylS family or to the two-component response regulator families. Alternative sigma factors and histone like proteins were also involved in PAI regulation.Dogmatic cascades, in which PAI encoded regulators of PAI located virulence genes are modulated by systems and encoded outer surface of PAI, including VPI of *Vibrio cholerae*, SPI-1 and SPI-2 of *S. enterica*, the Yop virulon of pathogenic *Yersinia* spp., and the LEE of enteropathogenic *E. coli* (EPEC) and EHEC. The parameter of SPI-1, SPI-2, and LEE of *E. coli* has been investigated in a number of examine. Even though many features are not yet found (Fig. 2.1). As an example, we briefly illustrate the regulation of offensive genes of *S. enterica* SPI-1. SPI-1 genes are expressed under conditions obligatory on the pathogen by the host microenvironment. Such circumstances include: the oxygen rank, osmolarity process, bacterial growth stage, pH value and as newly described, the occurrence of short chained unstable fatty acids (Durant et al., 2000). Conditions of low oxygen and elevated osmolarity induce invasiveness, whereas under high-oxygen condition, the bacteria remain non invasive. The transduction of these signals may be reliant on the function of the two-component global regulatory systems EnvZ/OmpR, BarA/SirA, PhoPQ, and PhoRB, as well as on FliZ and Hha, all encoded by genes on the core genome.

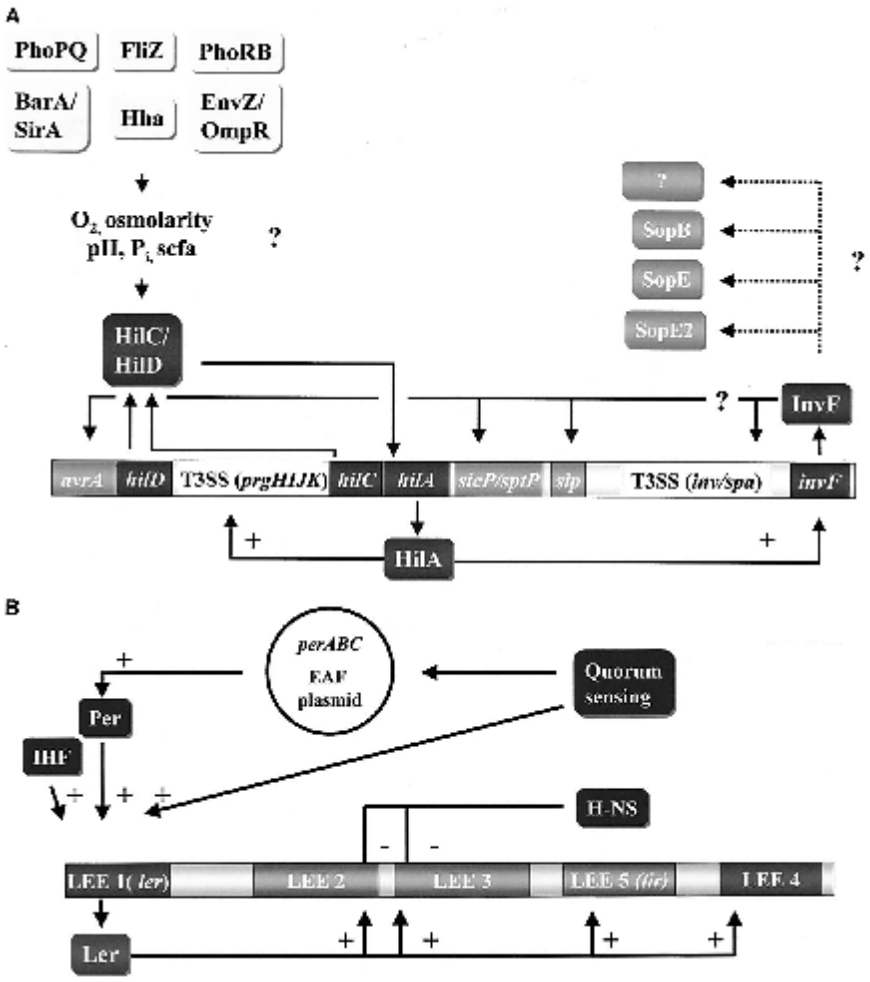


Fig. 2.1. Regulation of *S. enterica* SPI-1 and LEE of EPEC.

(A) SPI-1 of *S. enterica* encodes a number of transcriptional regulators. Current genetic evidence is most consistent with a cascade of transcriptional activation in which HilD/HilC, HilA, and InvF (dark grey bars) act in sequence to activate SPI-1 genes. First, HilD and HilC bind to several sites within P*hilA* and derepress *hilA* transcription. Then HilA binds to *invF* and *prgH* transcription start sites and activates the expression of *invD* and *prgH*. This results in expression of the genes encoding the T3SS (white bars). InvF is also required for expression of sptP, so it is possible that sicP sptP may be co- transcribed with the *sip* genes. Two other SPI-1 effectors, SigD (SopB), SopE, SopE2, and other unnamed factors are also expressed from InvF/SicA-

dependent promoters. Whereas the HilD-HilA-InvF cascade is most probable, deviations may occur. A number of environmental signals such as oxygen, osmolarity, growth stage, bile salts, and short-chain fatty acids have been described to alter SPI-1 expression, probably dependent on the function of the component regulatory systems EnvZ-OmpR, BarA-SirA, PhoPQ, and PhoRB as well as FliZ, and Hha. (B) LEE1, LEE2, and LEE3 (light grey bars) represent three polycistronic operons encoding the T3SS. LEE4 (grey bar) encodes the secreted LEE effectors, and LEE5 (dark grey bar) encodes Intiman and Tir. The first gene of LEE1 is *ler*, encod in a regulatory protein which is part of the regulatory cascade. Ler activates LEE2, LEE3, LEE4, and LEE5 expression. LEE1 is not synchronized by Ler. The expression of *ler* itself is regulated by the plasmid encoded regulator Per, which is encoded by the *per ABC* operon. Per-mediated regulation of LEE is modulating due to special environmental signals. Expression of LEE genes is also reliant on the histone like proteins, H-NS, that usually down regulate gene here it down regulate the LEE2 and LEE3 operons. LEE is also regulated by IHF, a global regulator which was essential for *ler* appearance. Molecules that are fashioned by the quorum-sensing machinery stimulate LEE1 and LEE2 operons. Up regulation of LEE1, in turn increases the expression of LEE3 and LEE4.

3. OBJECTIVE

Testing for individual disease causing agents are possible and is often done when there is a known or suspected occurrence of a waterborne disease. However in some cases officially unreasonable, to routinely observe for all disease causing bacteria, viruses, and protozoas that may be found in impure surface water. For routine water quality monitoring, nontoxic bacteria that occur in higher figures and originate from the same sources as the diseases causing bacteria are typically calculated. Even though the presence of indicator bacteria does not demonstrate that pathogenic bacteria are present in the environment, the presence does explain the contamination by fecal material has occurred. Elevated concentration of microbial indicators and concentrations that beat standards demonstrate an increased risk of exposure to injurious bacteria and the associated undesirable effects. Vedvyas and Koel rivers present in Rourkela, Odisha are two important river from domestic utilization point of view. The slum areas as well as urban areas consume the water from both the rivers either directly or in an distilled manner. It is a common mode of practice in these locations for the cattle to use these water bodies for their livelihood. Hence any mode of pollution at any level puts a direct impact on the populations. Keeping in mind the above situation and importance of monitoring the pollution level of the two rivers from indicator organism point of view, the present work was carried out with the following objectives:

- To enumerate coliform bacterial population in domestic waste polluted water sample by employing MPN test method.
- Isolation of coliform bacteria from domestic waste polluted water sample.
- Screening of *E. coli* which having pathogenic nature.
- Antibiotic sensitive test of the pathogenic *E.coli* environmental isolate.
- Biochemical test of the isolates.
- Identification of number and type of PAI responsible for the pathogenicity nature of positive strain in *E. coli*.

4. MATERIALS AND METHODS

4.1. Collection of Sample

Water sample were collected from the study sites (Vedvyas river and Koel River) which have been polluted with the domestic wastes generated from the inhabitants of the Steel city, Rourkela. As the river bank of Vedvyas is a cremation point hence the chances of domestic pollution is more in this study location. The water samples were collected aseptically in autoclaved sample container and were transported to the laboratory by keeping them on ice box. The samples were processed immediately for the determination of coliform bacterial populations count.

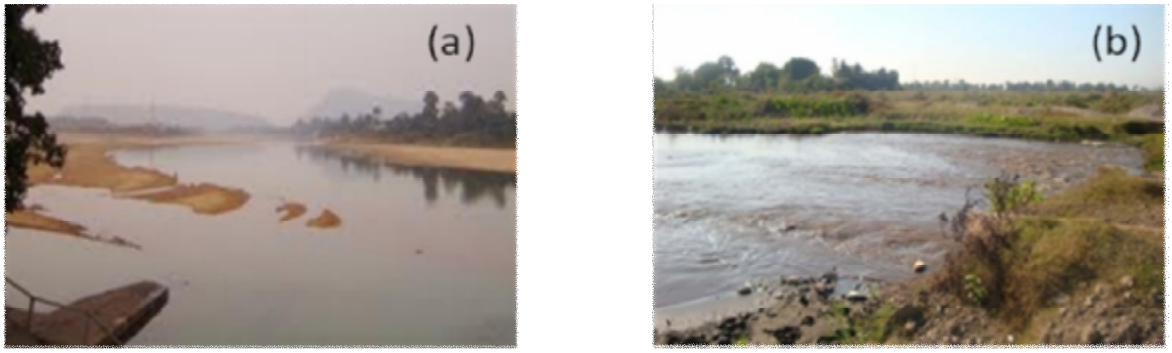


Fig. 4.1. Sites of sample collection (a) Vedvyas River (b) Koel River

4.2. Total Coliform count by Most Probable Number (MPN) Test

In order to assess the domestic pollution level in the study sites the Most Probable Number (MPN) Test was conducted by using the coliform bacterial populations as an indicator organism (Cappuccino and Sherman, 2008). The test was done as follows by using :

4.2.1. Presumptive test

A set of three Lauryl Tryptose broth [Tryptose-20g, Lactose-5g, NaCl-5g, K_2PO_4-2.75g, KPO_4-2.75g, Sod Lauryl Sulphate-0.10g, pH-6.8±0.2, 1000mL Distilled water] containing 10 mL of five double strength and ten single strength were prepared and autoclaved by putting Durham's tube in them. 10 mL of water sample was inoculated in to the double strength tubes and 1 mL

and 0.1 mL sample was inoculated in to the second five single strength tubes and third set of five single strength tubes respectively. The tubes were incubated at 37ºC for overnight. The result was noted for the gas formation in the tubes and growth in the tubes by appearance of turbidity in the tubes. If growth and gas are seen in the tube after incubation for up to 2 days at 35°C, one can presume that at least one coliform was originally inoculated into the medium, multiplying into a large population of cells while fermenting lactose with the production of acid and gas.

The number of positive tubes was counted in each set of tubes and the result was compared with the standard chart to determine the Most Probable Number (MPN) of the sample.

4.2.2. Confirmatory test

Each positive tube (i.e., showing growth and gas) was then inoculated into a tube each of two media for the Confirmatory Test. These media were strongly selective for gram-negative organisms and may even inhibit some enterics. Growth and gas form a positive result as for McConkey agar [Peptone-24.50g, Lactose-10g, Synthetic detergents-0.50g, Neutral Red-0.04g, Agar-20g, pH-7.4Peptone-24.50g, Lactose-10g, Synthetic detergents-0.50g, Neutral Red-0.04g, pH-7.4±0.2, 1000mL Distilled Water].

For the selective enrichment and detection of the fecal coliforms, the samples from positive samples were streaked on the Eosin Methylene Blue (EMB) plates [Peptic digest of animal tissue-10g ,K_2PO_4-2g, Lactose-5g, Eosin-Y-0.40g, Ethylene blue-0.065g, Agar-13.50g, pH- 7.2 ± 0.2, 1000 mL Distilled Water] and the peculiar characteristic feature of coliform bacteria were observed for greenish metallic sheen in the bacterial colony. If the characteristics are found to be present in the isolated samples then the presence of coliform bacteria is confirmed in the water sample.

4.2.3. Completed test

The positive samples from the EMB plates were streaked and re-streaked on Luria Agar [0.5% peptone, 0.5% yeast extract, 1% NaCl, 1.5% Agar, at pH 7.5±0.2, 1000mL Distilled Water] plates for the isolation of pure culture and the biochemical characterization of the isolates were conducted. The isolated pure cultures were then inoculated in to the freshly prepared Lauryl

Tryptose broth and the test was repeated to confirm that the isolated coliform bacteria was responsible for the positive test in presumptive test thus completing the completed test.

A total of eight numbers of coliform bacteria were isolated from the two study sites responsible for the positive tests in the MPN test.

4.3. Total Coliform Count by Membrane Filtration Technique

The Membrane Filtration Technique is an effective, accepted technique for testing liquid samples for microbiological contamination. It is the direct method for counting microorganisms which involves less preparation than other traditional methods. The advantage in Membrane Filtration Technique is that the presence or absence result can be obtained within 24 hours of inoculation in EMB agar plate. In this technique 100 mL of the collected water sample was filter with 0.22µm size filter paper by using vacuum filtration system and the filter paper was placed on to the prepared EMB plates which were incubated for 48 hours and the number of colonies was counted using the colony counter (Taras ***et al.***, 1998).

4.4. Phenotypic Characterization of the Isolates

The phenotypic characterizations of the isolates were conducted by observing the colony morphology of on McConkey Agar plates and Luria Agar plates. The cell morphology was analyzed using oil immersion microscope with 100X magnification.

4.5. To study the nature of the environmental isolates on the basis of their pathogenicity

The pathogenicity nature of the environmental isolates was tested by streaking the isolates on Blood Agar plates [Meat extract-10.0g, Peptone-10.0g, and NaCl-5.0 g, Agar-15.0 g, and pH 7.3±0.2, Distilled Water-1000 mL]. The plates were incubated for 48 hr at 37ºC and the clear zone was observed for haemolysis of Red Blood Cells (RBC) by the bacterial isolate which confirms their pathogenic nature.

4.6. Biochemical Characterization of the isolates

The biochemical characteristics of the isolates were conducted by using HiMedia Rapid Biochemical Identification kit [KB003 Hi25®]. The test includes certain amino acid utilization and sugar utilization tests.

4.7. Antibiotic Sensitivity Pattern of the isolates

All the isolates were tested for antimicrobial resistance by the method of (Bauer *et al.*, 1966) with antibiotic impregnated discs (Hi-Media Laboratory, Bombay, India). The following antibiotic discs with conc. of the drug as stated in the observation were used, Amoxycillin (AM, 30µg), Vancomycin (VA, 30µg), Chloramphenicol (C, 30µg), Tetracyclin (TE, 30µg), Norfloxacin (NX, 10µg), Neomycin (N, 30µg), Clavulanic acid (AC, 30µg) and Kanamycin (K, 20µg). The antibiotic test was performed as per CLSI guidelines (CLSI, 2006) which have been described as follows:

Mueller Hinton Agar [Hiveg infusion-2g, Hiveg acid hydrolysate-17.50g, Starch-1.50g, Agar-17g, pH-7.3±0.2, 1000mL Distilled Water] plates were prepared for testing the antibiotic susceptibility of the isolated strains. Pure cultures were used as inoculums. The colonies were transfer to Lueria Bertani (LB) broth (5 ml), incubated at 35 ± 2^0C till the development of mordent turbidity. Suitable turbidity of about 0.5 McFarland was reached by diluting the growth of the culture inoculums and a sterile cotton swab was dipped into the inoculum in the broth and rotated firmly against the upper inside wall of the tube to express excess fluid. The entire agar surface was streaked with the swab for 3 times turning the plate at 60^o angle between each streaking. The inoculum was allowed to dry for 5 min. The discs were dispensed using aseptic technique at least 24 mm apart. Petri plates were incubated immediately at 37^0C and examined after 16-18 hours. The zones showing complete inhibition were measured and the diameters of the zones were measured to the nearest millimetre. By the antibiotic zone scale, the area of inhibition was measured for each antibiotic. Sensitivity of the isolates to each antibiotic was determined according to the chart provided by Himedia, Mumbai.

4.8. To study the genetic mechanism of pathogenicity in contrast to the presence of Pathogenicity Islands (PAIs)

Amplification of *PAI* gene was performed by the method of Warren *et al.*,1997 using pairs of 697 (59-TGTCGGCGTTCGTTGTC-39) and 698(59-CTGGTGCTGGGGCTATT-39), using 25 cycles of 94°C for 2 min, 53°C for30 s, 72°C for 40 s, 94°C for 30 s, and 72°C for 3 min. The right junction was amplified with primers 682 (59-CCAGCAGGAAGTTGCGGA-39) and 684 (59-AACACCATCTTTGCGCTTT-39). Each of the reaction mixtures contained 2.5µl of 10X PCR amplification buffer [500 mM KCl, 100 mM Tris-HCl (pH 9.0), 1.0% Triton X-100], 1.5 µl of $MgCl_2$ (25 mM), 0.5µl each of 10 mM dATP, dCTP, dGTP and d TTP, 0.5µl each of forward (PAI-F) and reverse (PAI-R) primers for PAI, 1 µl of Taq DNA polymerase at 2.5 U/µl; and sterile Milli-Q water to make a final volume of 21µl and 4µl of template DNA (50ng/µl). PCR was programmed as follows: an initial denaturation at 94°C for 2 min., followed by using 25 cycles of 94°C for 2 min, 52°C for30 s, 72°C for 100 s, 94°C for 30 s, and 72°C for 3 min. PCR was carried out in a thermal cycler (Bio-Rad Inc., USA) using 200µl PCR tubes with a reaction mixture volume of 25µl. The size of the PCR product was ascertained by electrophoresis in agarose 1% gel and visualized in a gel documentation system (Gel Doc XR, Bio-Rad, USA).

5. RESULTS

5.1. Enumeration of Coliform bacterial population by Most Probable method (MPN)

After performing the MPN test the coliform bacterial populations in the two study sites were found to be in the range of seven for both Vedvyas and Koel river site by comparing the value of the positive test tubes (Fig. 5.1 a and b) with the standard chart as provided by Taras et al., 1998.

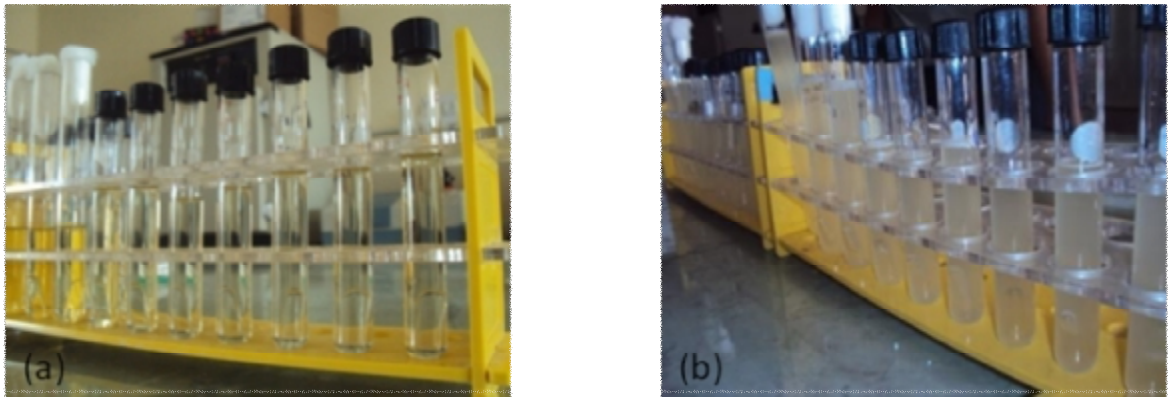


Fig. 5.1. MPN test result of the two study sites (a) those showing negative results and (b) those showing positive results

5.1.1. Confirmation of Coliform bacteria by streaking on EMB plates

When the positive tubes from the MPN test were streaked on EMB plates the coliform characteristic features of greenish metallic sheen (Fig. 5.2 a and b) was found in the isolated colonies, thus confirming the presence of coliform bacteria in the test sample.

5.1.2. Pure cultures of positive isolates on McConkey Agar and Luria Agar Plates

The positive isolates giving greenish metallic sheen on EMB Agar plates were repeatedly streaked on McConkey Agar and Luria Agar plates and the colony morphology was determined by phenotyping observation which has been shown in Table 1 and Fig. 5.3 and Fig. 5.4.

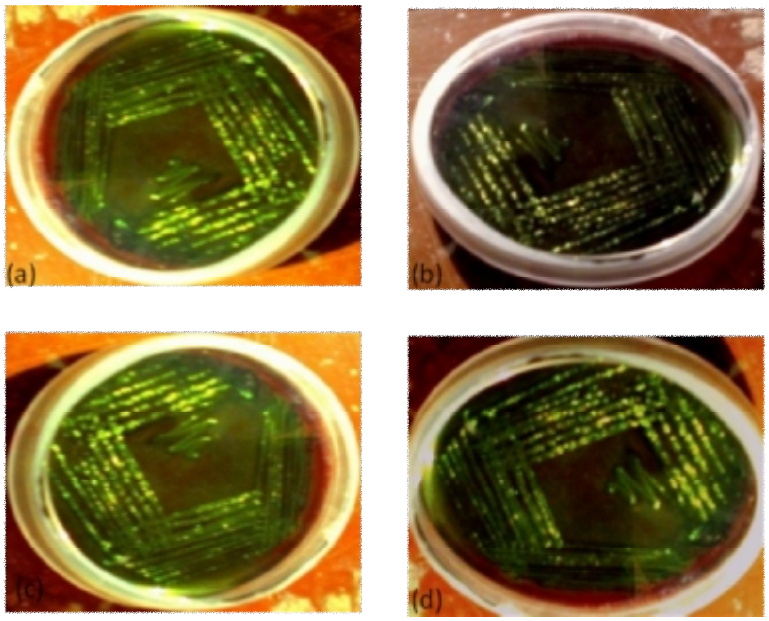


Fig. 5.2 (a) Bacterial isolates showing positive results on EMB Agar plates (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) KDS-4.

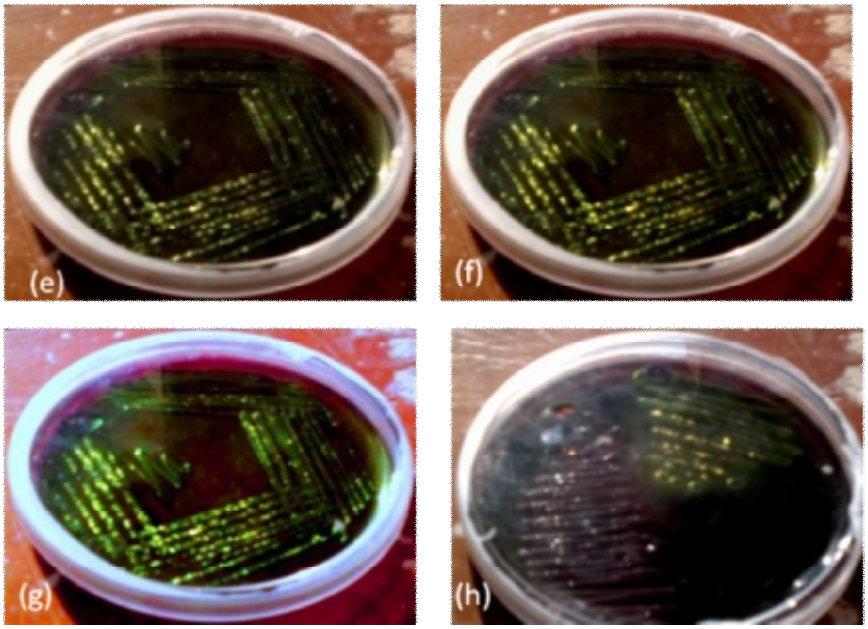


Fig. 5.2 (b) Bacterial isolates showing positive results on EMB Agar plates (e) VSS-5, (f) VDS-6, (g) VDS-7, (h) VDS-8.

Table 1. Colony morphology of the positive isolates on different media

Isolate #	Colony morphology	
	McConkey Agar Plates	**Luria Agar Plates**
KSS-1	Pink, rounded, opaque	White, oval out line, elevated
KSS-2	Pink, rounded, opaque	White, oval out line, elevated
KDS-3	Pink, rounded, opaque	White, oval out line, elevated
KDS-4	Pink, rounded, opaque	White, oval out line, elevated
VSS-5	Pink, rounded, opaque	White, oval out line, elevated
VDS-6	Pink, oval, opaque	White, oval out line, elevated
VDS-7	Pink, rounded, opaque	White, oval out line, elevated
VDS-8	Purple coloured, oval, opaque, slimy	Rough outline, white, transleucent

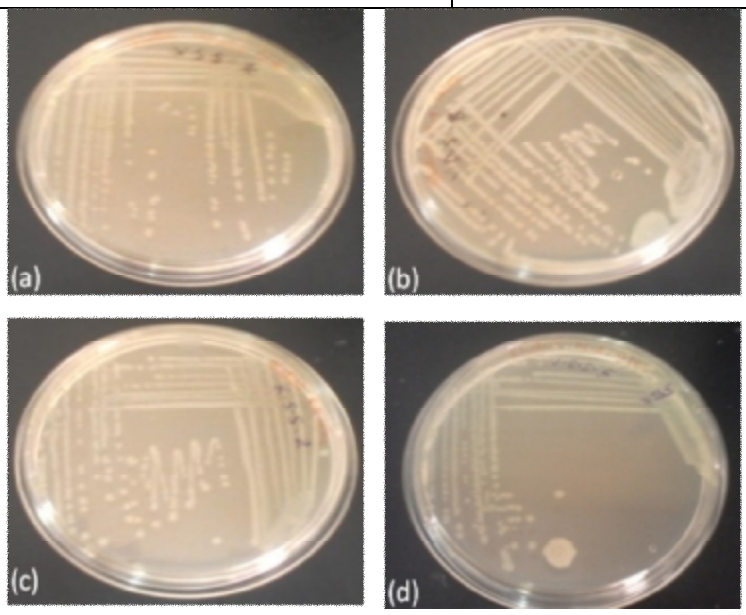


Fig. 5.3. a. Colony morphology of positive isolates on Luria Agar plates (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) KDS-4.

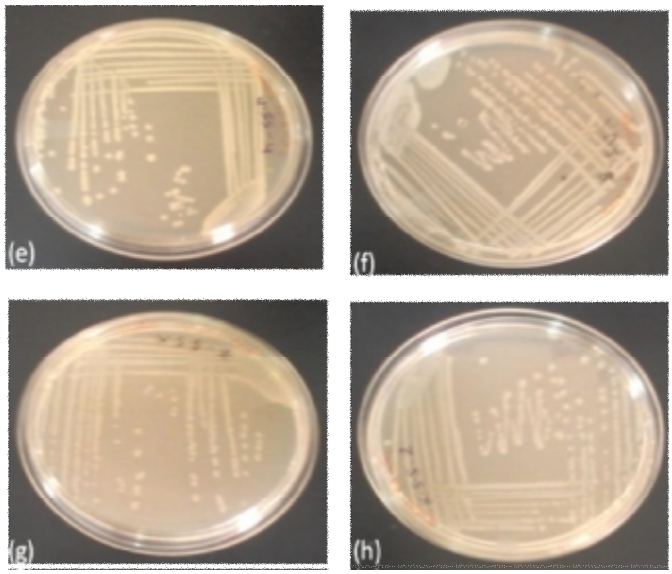


Fig. 5.3.b. Colony morphology of positive isolates on Luria Agar plates (e) VSS-5, (f) VDS-6, (g) VDS-7, (h) VDS-8.

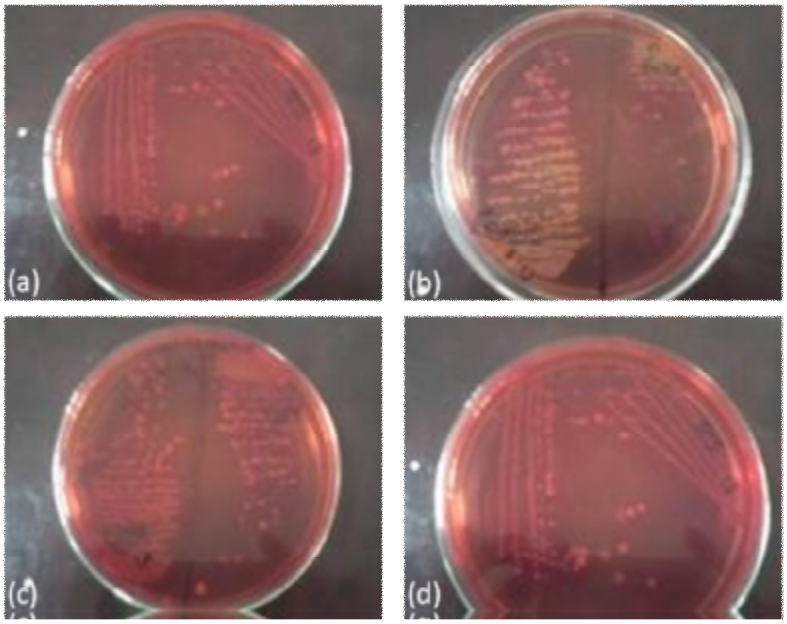


Fig. 5.4.a. Colony morphology of positive isolates on McConkey Agar plates (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) KDS-4.

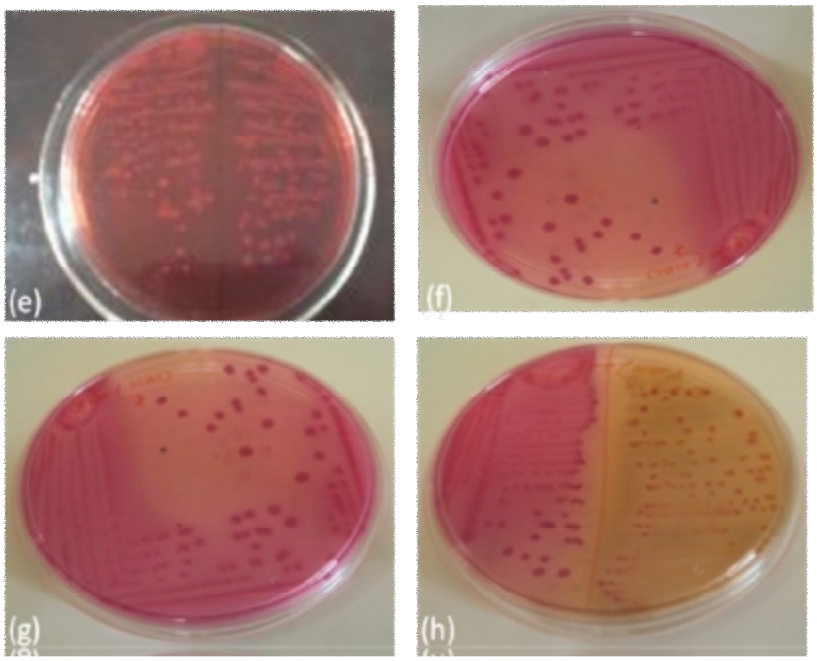


Fig. 5.4.b. Colony morphology of positive isolates on McConkey Agar plates (e) VSS-5, (f) VDS-6, (g) VDS-7, (h) VDS-8.

5.2. Total Coliform Count in the sample by Membrane Filtration Technique

The two water samples collected from Vedvyas and Koel river were used for this study. In Vedvyas sample the total number of colonies present in filter paper was 84 CFU per 100 ml and in Koel river sample it was found to be 68 CFU per 100ml. The result of which has been shown below in the Figure 4.6 a and b.

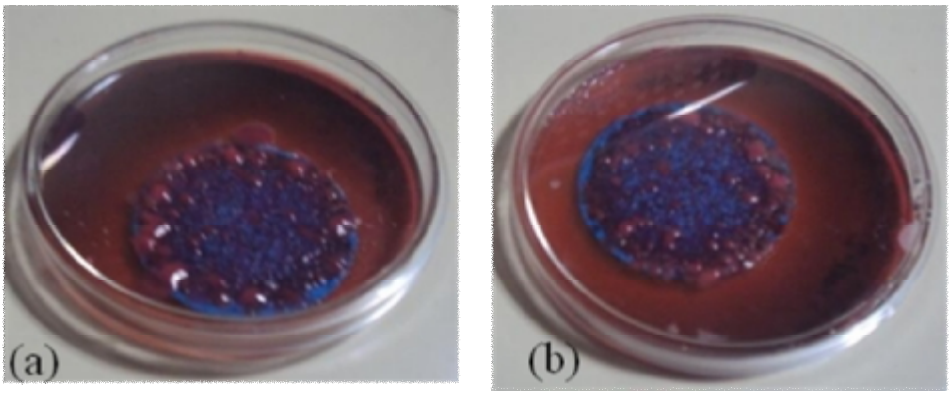


Fig. 5.5. Membrane Filter Technique a) Koel river, b) Vedvyas river

5.3. Gram staining of the isolates

All the eight positive isolates except one gave the result of gram negative which is the characteristic feature of E. coli and the cell morphology were the same like that of E. coli under oil immersion microscope. The Gram's staning result s has been shown in Table 2 as well as in Fig. 5.6.

Table 2. Gram Staining result of the positive isolates

Isolate #	Gram's Staining Result	Cell Morphology
KSS-1	-ve	Bacillus
KSS-2	-ve	Bacillus
KDS-3	-ve	Bacillus
KDS-4	-ve	Bacillus
VSS-5	-ve	Bacillus
VDS-6	-ve	Bacillus
VDS-7	-ve	Bacillus
VDS-8	+ve	Bacillus

5.4. RBC Haemolysis test

Out of eight positive isolates in EMB agar, when the haemolysis test was conducted using Blood Agar plates to know whether the environmental isolates harbour pathogenic nature or not, out of eight six isolates gave positive result. Thus, the environmental coliform isolates also possess pathogenic nature and can cause potential hazard to the fauna using those water for their daily life. The result of the blood haemolysis test has been providing in the Table 3 and Fig. 5.7.

5.5. Biochemical Characterization of the Pathogenic Environmental Isolates

The pathogenic environmental isolates gave a same pattern of their biochemical characteristics utilizing the same sugar molecules and aminoacids. The detailed result has been provided in Table 4 as well as Fig. 5.8.

5.6. Antibiotic Sensitivity pattern of the isolates

Though the isolates were of environmental in origin but due to huge contamination of domestic wastes the isolates were resistant to a number of antibiotics and the result of which has been shown in Fig. 5.9 and Table 5.

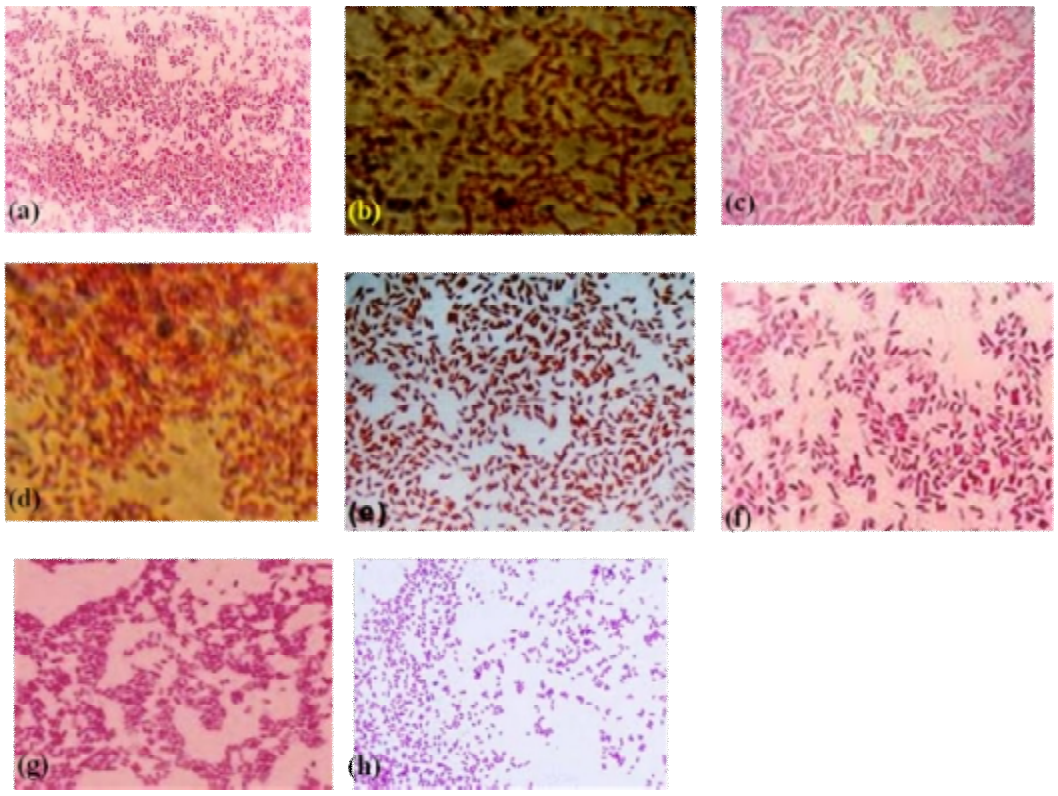


Fig 5.6. Physical Characterization Of Bacteria (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) KDS-4, (e) VSS-5, (f) VDS-6, (g) VDS-7, (h) VDS-8.

Table 3. Isolation of strain on the basis of their pathogenicity

Strain number	Blood Agar Result
KSS-1	+ve
KSS-2	+ve
KDS-3	+ve
KDS-4	-ve
VSS-5	+ve
VDS-6	+ve
VDS-7	+ve
VDS-8	-ve

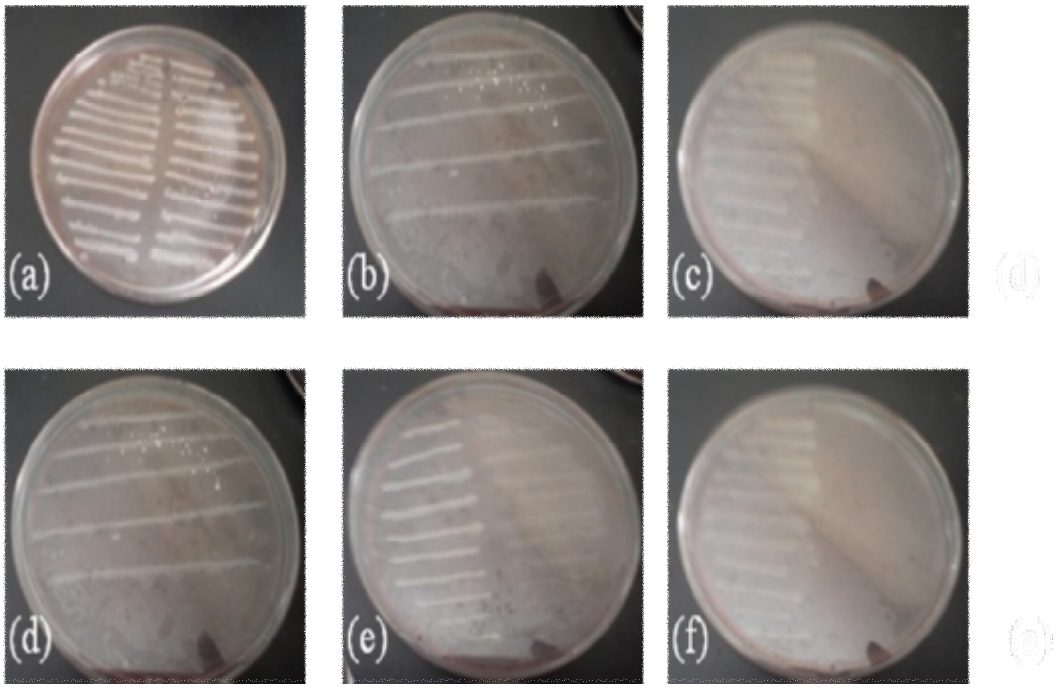


Fig. 5.7. Screening of E-coli which having pathogenic nature (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) VSS-5, (e) VDS-6, (f) VDS-7.

Table 4. Biochemical characteristic of the pathogenic environmental isolates

Sl. No.	Tests Conducted	VDS-6	VSS-5	KDS-3	VDS-7	KSS-1	KSS-2
1	Lactose	+	+	+	+	+	+
2	Xylose	+	+	+	+	+	+
3	Maltose	+	+	+	+	+	+
4	Fructose	+	+	+	+	+	+
5	Dextrose	+	+	+	+	+	+
6	Galactose	+	+	+	+	+	+
7	Rafinose	+	+	+	+	+	+
8	Trehalose	+	+	+	+	+	+
9	Malibose	+	+	+	+	+	+
10	Sucrose	+	+	+	+	+	+
11	L.Arbinose	-	+	+	+	+	+
12	Mannose	+	+	+	+	+	+
13	Inulin	-	-	-	-	-	-
14	Sodium gluconate	-	-	-	+	+	+
15	Glycerol	-	+	-	+	+	+
16	Salicin	+	+	+	+	+	+
17	Dulcitol	-	+	+	+	+	+
18	Inositol	-	-	-	-	+	+
19	Sarbitol	-	+	+	-	+	+
20	Mannitol	+	+	+	+	-	+
21	Adonitol	-	+	+	-	-	-
22	Arbitol	-	+	+	-	-	-
23	Erythritol	-	-	-	-	-	-
24	Alpha-Methyl-d-glucoside	-	-	-	-	-	-
25	Rhamnose	-	+	+	-	+/-	+
26	Cellobiose	+	+	+	+	+	+
27	Melezitose	-	+	-	-	-	-
28	Alpha-Methyl-d-mannoside	-	-	-	-	-	-
29	Xylitol	-	-	+	+	+	+
30	ONPG	-	+	-	+	+	+
31	Esculinhydrolysis	+	+	+	+	+	+
32	D-Arbinose	-	-	+	+	-	+
33	Citrate utilization	+	+	+	+	+	+
34	Malonate utilization	+	+	+	+	-	+
35	Sarbose	-	-	-	-	-	-

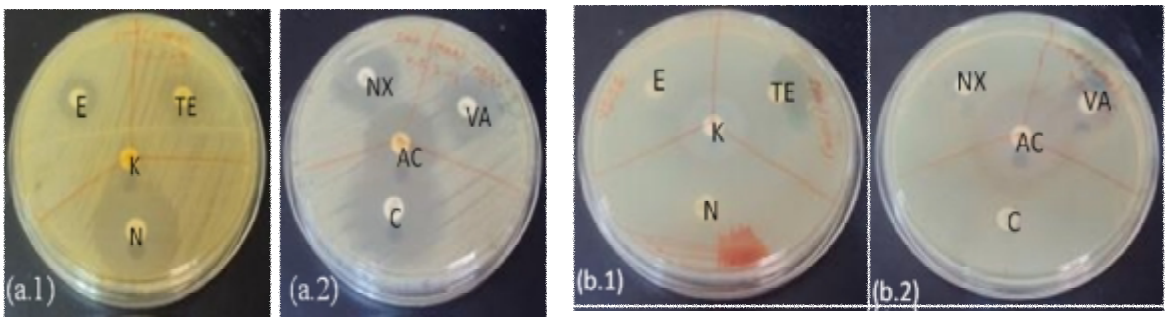
Fig. 4.8 Biochemical Characters of the pathogenic environmental isolates (a) KSS-1, (b) KSS-2, (c) KDS-3, (d) VSS-5,(e) VDS-6,(f) VDS-7

Table 5. Antibiotic sensitive test

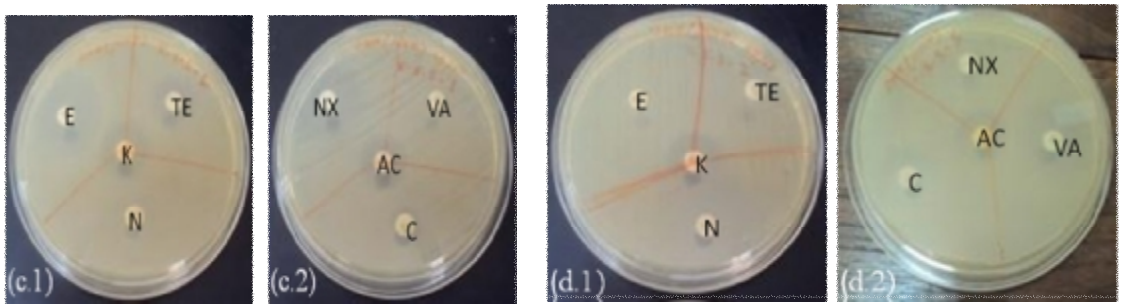
Isolate #	Zone Diameter of Antibiotics (mm)								Antibiogram
	E	TE	N	NX	K	VA	AC	C	
KSS-1	19 (I)	17 (I)	30 (S)	18 S)	-	12 (R)	12 (R)	20 (S)	$E^{R}TE^{I}N^{I}NX^{S}K^{S}VA^{R}AC^{R}C^{S}$
KSS-2	10 (R)	15 (I)	15 (I)	20 (S)	19 (S)	-	8 (R)	21 (S)	$E^{R}TE^{I}N^{I}NX^{S}K^{S}VA^{R}AC^{R}C^{S}$
KDS-3	13 (R)	9 (R)	15 (I)	28 (S)	18 (S)	-	-	14 (I)	$E^{R}TE^{R}N^{I}NX^{S}K^{S}VA^{R}AC^{R}C^{I}$
VSS-5	12 (R)	16 (I)	19 (S)	25 (S)	17 (I)	-	8 (R)	21 (S)	$E^{R}TE^{I}N^{S}NX^{S}K^{I}VA^{R}AC^{R}C^{S}$
VDS-6	15 (R)	10 (R)	17 (S)	28 (S)	7 (R)	-	-	20 (S)	$E^{R}TE^{R}N^{S}NX^{S}K^{R}VA^{R}AC^{R}C^{S}$
VDS-7	13 (R)	18 (I)	17 (S)	24 (S)	19 (S)	11 (R)	11 (R)	22 (S)	$E^{R}TE^{I}N^{S}NX^{S}K^{S}VA^{R}AC^{R}C^{S}$

E-Erythromycin ,TE- Tetracyclin, N- Neomycin, NX- Norfloxacin, K- Kanamycin, VA-Vancomycin, AC-Amoxyclav, C- Chloramphenicol.

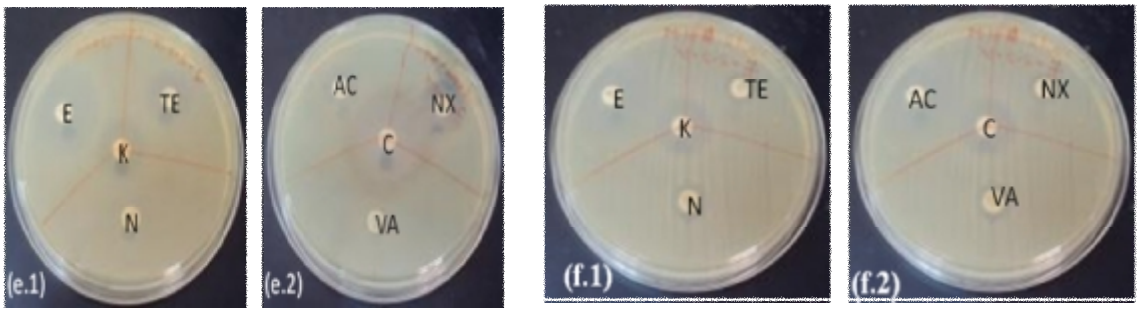
R- Resistance, **S**- Sensitive, **I**- Intermediate



(a) KSS-1 (b) KSS-2



(c) KDS-3 (d) VSS-5



(e) VDS-6 (f) VDS-7

Fig. 5.9. Zone of inhibition by performing antibiotic sensitive test

5.7. Amplification of PAI in the pathogenic environmental isolates

A clear distinct banding pattern was observed in PCR when PAI was amplified in the isolated environmental isolates. An amplicon size of around 330 bp (Fig. 5.10) was observed in five out of eight environmental isolates. Hence the nature of pathogenicity in those bacteria is confirmed due to the presence of pathogenicity islands.

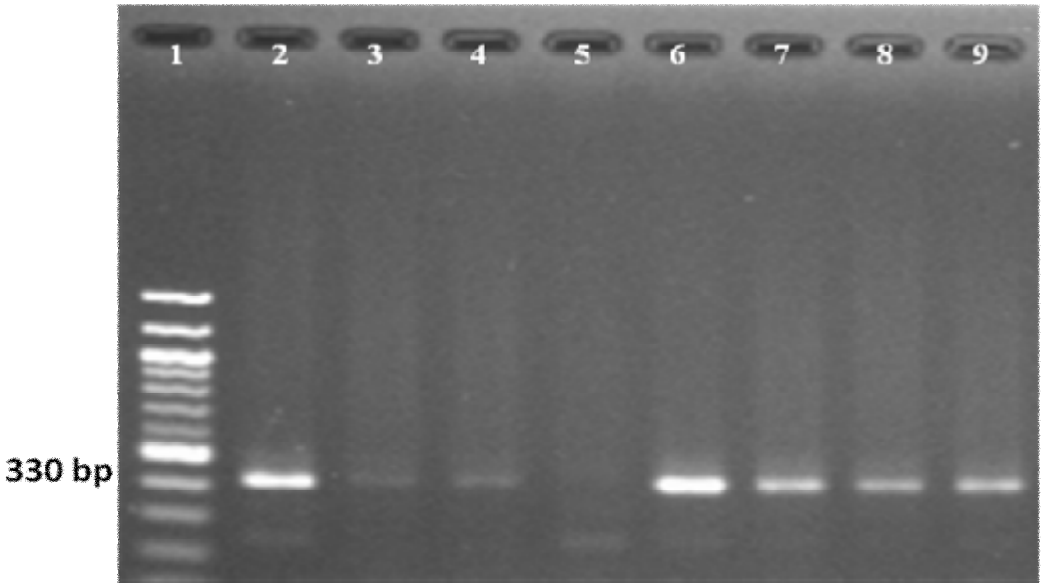


Fig. 5.10. Amplification of PAI gene in the isolate bacterial strains lane-1 100bp ladder, lane 2: KSS-1, lane3: KSS-2, lane4: KDS-3, lane5: KDS-4, lane6: VSS-5, lane7: VDS-6, lane8: VDS-7, lane9: VDS-8.

6. DISCUSSION

Water born diseases cause serious health associated problems in developing countries. Coliforms are the indicators to assess the domestic pollution level in water samples. MPN test is a primitive step to identify the presence or absence of *E.coli* in water sample. In present study, we have determined a total of seven numbers of coliform bacteria present in Vedvyas and Koel river where as in Ganges River Varanasi the count is much higher in the range of 10^7 CFU per 100 ml **(**Mishra et al., 2006). The number of total coliforms in the test samples by Membrane Filtration Technique was very high well above the permissible limit of coliforms in the water sample for domestic use. The value is higher than the other domestic polluted rivers like Ganga and Yamuna **(**Mishra et al., 2006; Zeyaullah et al., 2010). When the isolated coliforms were inoculated into MacConkey agar plates pink colouration developes which is the characteristic features of the gram negative coliform bacteria. On EMB plates the organisms developed greenish metallic sheen after giving 24 hrs of incubation which is the unique characteristic feature of *E. coli* (Zinnah et al., 2007). When gram staining was performed for physical characterization of bacteria, out of eight six isolates i.e., KSS-1, KSS-2, KSS-3, KSS-4, VSS-5,VSS-7 showed round in shape and gram negative where as two strain i.e., VDS-6,VDS-8 showd oval shape and VDS-6 is gram negative and VDS-8 is gram positive bacteria. The biochemical characteristics of the isolates on HiMedia Rapid Biochemical Identification kit [KB003 Hi25®] showed promising results for the identification of the environmental isolates. The test includes certain aminoacid utilization and sugar utilization tests. All eight isolates shown positive result towards Xylose, Dextrose, Galactose, Mannose, Cellobiose, Esculin hydrolysis, Citrate utilization and all of them showed negative towards Inulin, Erythritol, Alpha-Methyl-d-glucoside, Alpha-Methyl-d-mannoside, Sarbose which is well accordance with the result obtained by Zeyaullah et al. (2010), the result of which are specific for environmental *E. coli* isolates. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the wafer indicating poor growth. Otherwise it shows resistance towards bacteria. In Vancomycin VSS-7 strain shows resistance property but other antibiotics shows no effect into it. The *E. coli* isolates from Ganges River Varanasi showed resistance towards, Erythromycin, Rifampicin, Gentamicin, Methicillin and Vancomycin, Gtreptomycin (Coque et al., 2005) which is also shown by our isolates in the present study. To see the

pathogenicity nature of the environmental isolates was tested by streaking the isolates on Blood Agar plates; the transparent zone was observed for haemolysis of Red Blood Cells (RBC) by the bacterial isolate which confirms their pathogenic nature. Akond et al. (2009) have also isolated many pathogenic *E. coli* isolates from the poultry environmental sample. Our result is also in accordance with the result obtained by Sheldon et al., 2010 who have found the environmental *E. coli* isolates to be pathogenic for cattle and mice. In order to know the genetic basis of pathogenesis in the environmental isolates the PAI gene was amplified in the environmental isolates. Out of eight isolates five gave a clear distinct banding pattern of 330 bp which confirms the presence of PAI in those isolates. The presence of PAI in uropathogenic *E. coli* has been demonstrated by Dobrindt et al. (2002) confirming the role of PAI in the pathogenic nature of E. coli isolates. Presence of PAI has also been confirmed in *E. coli* and *Citrobacter rodentium* causing haemorrhagic colitis and haemolytic–uraemic syndrome in humans which has been well established in the present study.

7. CONCLUSION

This work was mainly based on the isolation of coliform bacteria from river water as bacterial contamination cannot be detected by flavor, odor, or vision. Today, the MPN technique is most widely used for the enumeration of Coliforms in domestic water. This technique is simple to perform and inexpensive, it requires at least an overnight incubation period and a confirmation test process (24to48 additional hours) after the initial typical colony analysis. Additionally, when standard agar media are not used with this technique to recover stressed or injured Coliforms. The important challenges for the development of new coliform detection methods are to progress the specificity of the method, which could reduce the time consuming confirmation step, to take into account stressed and injured cells and to reduce the analysis time. Antibiotic susceptibility testing (AST) is usually carried out to determine and it is most successful in treating a bacterial infection *in vivo*. Small wafers containing antibiotics are placed onto a plate upon which bacteria are growing in selective media. If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is visualize around the wafer indicating poor growth, otherwise it shows resistance towards bacteria. PCR methods offer a higher rank of specificity detection. On the other hand, they have major limitations when applied to natural samples, including low amplification rates linked to the presence of inhibitor substances and the lack of information on the physiological activity of cells. The PCR method requires highly accomplished body as well as dedicated laboratory space and specific reagents to circumvent contamination of the samples by external DNA, particularly when nested PCR is required. In this study we determine the presence of coliform bacter it is easy to identify the group of microorganisms, the presence of bacteria in water were mainly leads to water born diseases. Here mainly we focus on the presence of Patogenicity Island in bacteria if it is present then it leads to dysentery, typhoid and other water born diseases some time it causes death. Water sample were collected from the study sites (Vedvyas river and Koel River) which have been polluted with the domestic wastes generated from the inhabitants of the Steel city, Rourkela. We found both the riverine systems of Rourkela were polluted by domestic sewage and by steel plant. From our resech we determine the total population of Coliform bacteria in Vedvyas is 84% and in Koel River it is 68%. However the most astonishing result is the presence of pathogenic strains in environmental conditions. 75% of the isolated bacteria showed positive result for RBC haemolysis confirming their pathogenic nature. When the genetic mechanism of pathogenicity was studied by amplification of PAI gene

83% of the pathogenic isolates confirmed the presence of PAI in their genome. Hence necessary corrective measures should be taken by the authorities to check the domestic pollutant discharge to the rivers to ensure the safe use of the river water for human and other live stocks.

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